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1. that I know well both the Japanese and English languages;
2. that the attached English translation is a true and correct translation of Japanese Patent Application No. 2000-115246 filed on April 17, 2000, priority of which is claimed in US Patent Application Serial No. 10/645,085 (Filing or 371 (c) Date: October 7, 2002), to the best of my knowledge and belief; and
3. that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 24, 2006

By:   
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Subaru Ref: FP1009PCT  
JPN Pat Appln No: 2000-115246

PATENT OFFICE  
JAPANESE GOVERNMENT

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Date of Application: April 17, 2000

Japanese Patent Application Number: 2000-115246

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Commissioner,

Patent Office

(Seal)

[Document] Patent Application  
[Case No.] DOJ-5404  
[Filing Date] April 17, 2000  
[To] Commissioner of the Patent Office Esq.  
[IPC]  
C07K 15/28  
C12N 5/16  
C12N 5/20  
C12N 15/06  
C12N 15/07  
C12N 15/09  
C12N 15/13  
C12P 21/08  
A61K 39/395

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[Designation of Official Fee]

[Ledger No.] 015565

[Amount of Payment] 21,000 yen

[Inventory of Submitted Documents]

[Article] Specification 1

[Article]	Abstract	1
[Article]	Drawing(s)	1
[General Power of Attorney Number]		0002667
[Proof]	Yes	

[Name of Document]	DESCRIPTION
[Title of Invention]	POLYPEPTIDE INDUCING APOPTOSIS
[CLAIMS]	

1. A reconstructed polypeptide which binds to Integrin Associated Protein (IAP), induces apoptosis of nucleated blood cells and causes no hemagglutination.
5. The reconstructed polypeptide of claim 1, wherein the reconstructed polypeptide is a modified antibody.
2. The reconstructed polypeptide of claim 1, wherein the modified antibody comprises two or more H chain V regions and two or more L chain V regions of the monoclonal antibody.
10. The reconstructed polypeptide of claim 3, wherein the reconstructed polypeptide is a dimer of a single-chain Fv comprising an H chain V region and an L chain V region.
15. The reconstructed polypeptide of claim 3, wherein the polypeptide is a single chain polypeptide comprising two H chain V regions and two L chain V regions.
6. A DNA encoding the single-chain Fv of claim 4.
20. 7. A DNA encoding the polypeptide of claim 5.
8. The reconstructed polypeptide of any one of claims 1 to 3, wherein the H chain V region and/or the L chain V region are humanized.
9. A DNA encoding the polypeptide of claim 8.
25. 10. An animal cell which produces the reconstructed polypeptide of any one of claims 1, 2, 3, 4, 5 and 8.

11. A microorganism which produces the reconstructed polypeptide of any one of claims 1, 2, 3, 4, 5 and 8.
12. A therapeutic agent for blood diseases which comprises the reconstructed polypeptide of one of claims 1,  
5 2, 3, 4, 5 and 8 as an active ingredient.
13. The therapeutic agent of claim 12 characterized in  
that the blood diseases is leukemia.
14. The therapeutic agent of claim 12 characterized in  
that the active ingredient is the single-chain Fv of claim  
10 4.

**[Detailed Description of the Invention]**

**[0001]**

**[Field of the Invention]**

A reconstructed polypeptide characterized by  
5 inducing apoptosis in nuclear blood cells having integrin  
associated protein (IAP) without causing hemagglutination.  
This reconstructed polypeptide contains at least two H chain  
V regions and at least two L chain V regions of a monoclonal  
antibody which induces apoptosis in nuclear blood cells  
10 having IAP. This reconstructed polypeptide is useful as a  
remedy for blood diseases mentioned below such as leukemia.

**[0002]**

**[Description of the Prior Art]**

The present inventors have made efforts to prepare  
15 a specific monoclonal antibody using a splenic stromal cell  
line as a sensitizing antigen aiming at developing specific  
antibodies that can recognize the aforementioned splenic  
stromal cells and succeeded in obtaining novel monoclonal  
antibodies that recognize mouse Integrin Associated Protein  
20 (mouse IAP) as an antigen. Then, the present inventors have  
further studied identities of said novel monoclonal  
antibodies using recombinant cells in which the mouse IAP  
gene had been introduced and discovered that the monoclonal  
antibodies are capable of inducing apoptosis of myeloid  
25 cells. (JPA 9-67499)

**[0003]**

The present inventors have succeeded in obtaining  
monoclonal antibodies whose antigen is human Integrin  
Associated Protein (hereinafter referred to as human IAP;  
amino acid sequence and nucleotide sequence thereof are  
5 described in J. Cell Biol., 123, 485-496, 1993; see also  
Journal of Cell Science, 108, 3419-3425, 1995) and which are  
capable of inducing apoptosis of human nucleated blood cells  
(myeloid cell and lymphocyte) having said human IAP. These  
monoclonal antibodies are referred to antibody MABL-1 and  
10 antibody MABL-2, and hybridomas producing these antibodies  
are also referred to MABL-1 (FERM BP-6100) and MABL-2 (FERM  
BP-6101), respectively. (WO99/12973)

**[0004]**

The present inventors made intensive research for  
utilizing the monoclonal antibodies against human IAP as  
therapeutic agent of blood diseases and obtained single  
chain Fvs having the single chain Fv region capable of  
inducing apoptosis of nucleated blood cells having human IAP.  
15 (JPA 11-63557)

20 **[0005]**

The monoclonal antibody recognizing IAP as an  
antigen induces apoptosis of nucleated blood cells having  
IAP, but it also causes hemagglutination in vitro. It  
indicates that the administration of a large amount of the  
25 monoclonal antibody recognizing IAP as an antigen may result  
in a side effect such as hemagglutination.

**[0006]**

**[Problem(s) to be Solved by the Invention]**

An object of this invention is to provide reconstructed polypeptides with improved property of inducing apoptosis of nucleated blood cells having Integrin Associated Protein (IAP) and with decreased or completely eliminated property of causing hemagglutination. Another object of the present invention is to provide therapeutic agents for blood diseases comprising the substance obtained as above which is capable of inducing apoptosis of nucleated blood cells having Integrin Associated Protein (IAP).

**[0007]**

**[Means for Solving the Problem]**

The present invention relates to the reconstructed polypeptides which binds to Integrin Associated Protein (IAP), induces apoptosis of nucleated blood cells having IAP and causes no hemagglutination.

**[0008]**

This invention also relates to reconstructed polypeptides which are modified antibodies.

**[0009]**

The modified antibodies of this invention can be any things which contain L chain V region and H chain V region of monoclonal antibody inducing apoptosis in nuclear blood cells having integrin associated protein (IAP), preferably human IAP, (e.g. antibody MABL- 1, antibody MABL- 2) and reconstructed polypeptides inducing apoptosis in nuclear blood cells having integrin associated protein (IAP),

preferably human IAP, without causing hemagglutination. Modified antibodies in which a part of amino acid sequence of V region has been altered are included.

**[0010]**

5                 The present invention also relates to the  
humanization of the above-mentioned reconstructed  
polypeptides. The humanized reconstructed polypeptides  
comprise a humanized H chain V region and/or a humanized L  
chain V region. Specifically, the humanized reconstructed  
10          polypeptides consist of the humanized L chain V region which  
comprises a framework region (FR) derived from an L chain V  
region of human monoclonal antibody and an CDR derived from  
an L chain V region of mouse monoclonal antibody and/or the  
humanized H chain V region which comprises an FR derived  
15          from an H chain V region of human monoclonal antibody and a  
CDR derived from an H chain V region of mouse monoclonal  
antibody. In this case, the amino acid sequence of FR or CDR  
may be partially altered, e.g. deleted, replaced or added.

**[0011]**

20          Furthermore, the present invention relates to  
reconstructed polypeptides inducing apoptosis in nuclear  
blood cells having integrin associated protein (IAP),  
preferably human IAP, which comprise an L chain C region of  
human monoclonal antibody and an L chain V region of the  
25          mouse monoclonal antibody, and/or an H chain C region of  
human monoclonal antibody and an H chain V region of the  
mouse monoclonal antibody.

[0012]

The present invention also relates to reconstructuted polypeptides inducing apoptosis in nuclear blood cells having integrin associated protein (IAP), preferably human IAP, which comprise a CDR derived from a monoclonal antibody of other mammals than mouse (such as human, rat, bovine, sheep, ape and the like), which is equivalent to said mouse CDR, or an H chain V region and an L chain V region containing the CDR. Such CDRs, H chain V regions and L chain V regions may include CDRs derived from a human monoclonal antibody prepared from, for example, a transgenic mouse or the like, and H chain V regions and L chain V regions derived from a human monoclonal antibody containing the CDR.

15 [0013]

The invention also relates to DNAs encoding the various reconstructed polypeptides as mentioned above and genetic engineering techniques for the producing recombinant vectors comprising the DNAs.

20 [0014]

The invention also relates to host cells transformed with the recombinant vectors. Examples of host cells are animal cells such as human cells, mouse cells or the like and microorganisms such as E. coli, Bacillus subtilis, yeast or the like.

25 [0015]

The invention relates to a process for producing the reconstructed polypeptides, which comprises culturing the above-mentioned hosts and extracting the reconstructed polypeptides from the culture thereof.

5 [0016]

The present invention relates to therapeutic agents for blood diseases comprising as an active ingredient the reconstructed polypeptide obtained in the above which induces apoptosis of nucleated blood cells having Integrin Associated Protein (IAP). The therapeutic agents for blood diseases of the invention are useful for the treatment of blood diseases, for example, leukemia such as acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, adult T-cell leukemia, multiple myeloma, mixed leukemia and hairy cell leukemia, malignant lymphoma (Hodgkin's disease, non-Hodgkin's lymphoma), aplastic anemia, myelodysplasia syndrome and polycythemia vera.

15 [0017]

20 [Mode for Carrying out the Invention]

The reconstructed polypeptides of the present invention preferably comprise two or more H chain V regions and two or more L chain V regions derived from monoclonal antibodies. The structure of the reconstructed polypeptides may be a dimer of single chain Fv comprising one H chain V region and one L chain V region or a polypeptide comprising 25 two H chain V regions and two L chain V regions. The

resulting reconstructed polypeptides contain variable regions of the parent antibodies and retain the complementarity determining region (CDR) thereof, and therefore bind to the antigen with the same specificity as that of the parent monoclonal antibodies.

5

**[0018]**

H chain V region

In the present invention, the H chain V region derived from a monoclonal antibody recognizes a cell surface molecule(s), for example, a protein (a receptor or a protein involved in signal transduction) or a sugar chain of the protein or on cell membrane and oligomerizes, for example, dimerizes through crosslinking of said molecule, and thereby serves as an agonist transducing a signal into the cells.

10 The H chain V region of the invention includes H chain V regions derived from a mammal (e.g. human, mouse, rat, bovine, sheep, ape etc.) and partially modified H chain V regions thereof. More preferable is a humanized H chain V region containing FR of H chain V region of a human monoclonal antibody and CDR of H chain V region of a mouse monoclonal antibody. The H chain V region further can be an H chain V region derived from a human monoclonal antibody corresponding to the aforementioned H chain V region of mouse monoclonal antibody, which can be produced by

15 recombination technique. The H chain V region of the invention may be a fragment of aforementioned H chain V

20

25

region, which fragment preserves the antigen binding capacity.

**[0019]**

L chain V region

5           In the present invention, the L chain V region derived from the monoclonal antibody recognizes a cell surface molecule(s), for example, a protein (a receptor or a protein involved in signal transduction) or a sugar chain of the protein or on cell membrane and oligomerizes, for  
10          example, dimerizes through crosslinking of said molecule, and thereby serves as an agonist transducing a signal into the cells. The L chain V region of the invention includes L chain V regions derived from a mammal (e.g. human, mouse, rat, bovine, sheep, ape etc.) and partially modified L chain  
15          V regions thereof. More preferable is a humanized L chain V region containing FR of L chain V region of human monoclonal antibody and CDR of L chain V region of mouse monoclonal antibodies. The L chain V regions further can be an L chain V region derived from human monoclonal antibody  
20          corresponding to the aforementioned L chain V region of mouse monoclonal antibody, which can be produced by recombination technique. The L chain V regions of the invention may be fragments of L chain V region, which fragments preserve the antigen binding capacity.

25

**[0020]**

Complementarity determining region (CDR)

Each V region of L chain and H chain forms an antigen-binding site. The variable region of the L and H chains is composed of comparatively conserved four common framework regions linked to three hypervariable regions or complementarity determining regions (CDR) (Kabat, E.A. et al., "Sequences of Protein of Immunological Interest", US Dept. Health and Human Services, 1983).

**[0021]**

Major portions in the four framework regions (FRs) form  $\beta$ -sheet structures and thus three CDRs form a loop. CDRs may form a part of the  $\beta$ -sheet structure in certain cases. The three CDRs are held sterically close position to each other by FR, which contributes to the formation of the antigen-binding site together with three CDRs.

**[0022]**

These CDRs can be identified by comparing the amino acid sequence of V region of the obtained antibody with known amino acid sequences of V regions of known antibodies according to the empirical rule in Kabat, E.A. et al., "Sequences of Protein of Immunological Interest".

**[0023]**

Single chain Fv

A single chain Fv is a polypeptide monomer comprising an H chain V region and an L chain V region linked each other which are derived from monoclonal antibodies. The resulting single chain Fvs contain variable regions of the parent monoclonal antibodies and preserve the

complementarity determining region thereof, and therefore  
the single chain Fvs bind to the antigen by the same  
specificity as that of the parent monoclonal antibodies (JP-  
Appl. 11-63557). A part of the variable region and/or CDR of  
5 the single chain Fv of the invention or a part of the amino  
acid sequence thereof may be partially altered, for example  
deleted, replaced or added. The H chain V region and L chain  
V region composing the single chain Fv of the invention are  
mentioned before and may be linked directly or through a  
10 linker, preferably a peptide linker. The constitution of the  
single chain Fv may be [H chain V region]-[L chain V region]  
or [L chain V region]-[H chain V region]. In the present  
invention, it is possible to make the single chain Fv to  
form a dimer, a trimer or a tetramer, from which the  
15 reconstructed polypeptide of the invention can be formed.

**[0024]**

Single chain reconstructed polypeptide

The single chain reconstructed polypeptides of the  
present invention comprising two or more H chain V regions  
20 and two or more L chain V regions, preferably each two to  
four, especially preferable each two comprise two or more H  
chain V regions and L chain V regions as mentioned above.  
Each region of the peptide should be arranged such that the  
modified single chain antibody forms a specific steric  
25 structure, concretely mimicking a steric structure formed by  
the dimer of single chain Fv. For instance, the V regions  
are arranged in the order of the following manner:

[H chain V region]-[L chain V region]-[H chain V region]-[L  
chain V region]; or  
[L chain V region]-[H chain V region]-[L chain V region]-[H  
chain V region],  
5 wherein these regions are connected through a peptide  
linker, respectively.

**[0025]**

Linker

In this invention, the linkers for the connection  
10 between the H chain V region and the L chain V region may be  
any peptide linker which can be introduced by the genetic  
engineering procedure or any linker chemically synthesized.  
For instance, linkers disclosed in literatures, e.g. Protein  
Engineering, 9(3), 299-305, 1996 may be used in the  
15 invention. If peptide linkers are required, the following  
are cited as example linkers:

Ser

Gly·Ser

Gly·Gly·Ser

20 Gly·Gly·Gly·Ser

Gly·Gly·Gly·Gly·Ser

Gly·Gly·Gly·Gly·Gly·Ser

Gly·Gly·Gly·Gly·Gly·Gly·Ser

(Gly·Gly·Gly·Gly·Ser) $n$

25 wherein n is an integer not less than one.

The method for introducing those linkers will be described in the explanation for DNA construction coding for modified antibodies of the invention.

**[0026]**

5       Preparation of reconstructed polypeptides

The reconstructed polypeptide binding to cells with human IAP are obtainable by connecting an H chain V region and an L chain V region derived from monoclonal antibodies against human IAP through the aforementioned linker. As examples of the single chain Fvs are cited MABL1-scFv and MABL2-scFv comprising the H chain V region and the L chain V region derived from the antibody MABL-1 and the antibody MABL-2, respectively.

**[0027]**

15       For the preparation of the polypeptide, a signal peptide may be attached to N-terminal of the polypeptide if the polypeptide is desired to be a secretory peptide. A well-known amino acid sequence useful for the purification of polypeptide such as the FLAG sequence may be attached for 20 the efficient purification of the polypeptide. The polypeptide can be efficiently purified with anti-FLAG antibody.

**[0028]**

25       For the preparation of the reconstructed polypeptide of the invention, it is necessary to obtain a DNA, i.e. a DNA encoding the single chain Fv or a DNA encoding reconstructed single chain polypeptide monomer.

These DNAs, especially for MABL1-scFv, and/or MABL2-scFv are obtainable from the DNAs encoding the H chain V region and the L chain V region derived from said Fv. They are also obtainable by PCR method using those DNA as a template and amplifying the part of DNA contained therein encoding desired amino acid sequence with the aid of a pair of primers corresponding to both ends thereof.

**[0029]**

In the case where each V region having partially modified amino acid sequence is desired, the V regions in which one or some amino acids are modified, i.e. deleted, replaced or added can be obtained by a procedure known in the art using PCR. A part of the amino acid sequence in the V region is preferably modified by the PCR known in the art in order to prepare the reconstructed polypeptide which is sufficiently active against the specific antigen.

**[0030]**

For the determination of primers for the PCR amplification, it is necessary to decide the type of the H chain and L chain of the antibody MABL-1 and/or the antibody MABL-2. It has been reported that the antibody MABL-1 has  $\kappa$  type L chains and  $\gamma_1$  type H chains and the antibody MABL-2 has  $\kappa$  type L chains and  $\gamma_2a$  type H chains (JP-Appl. 11-63557). For the PCR amplification of the DNA encoding the H chain and L chain of the antibody MABL-1 and/or the antibody MABL-2, primers described in Jones, S.T. et al., Bio/Technology, 9, 88-89, 1991 may be employed.

**[0031]**

For the amplification of the L chain V regions of the antibody MABL-1 and the antibody MABL-2 using the polymerase chain reaction (PCR), 5'-end and 3'-end oligonucleotide primers are decided as aforementioned. In the same manner, 5'-end and 3'-end oligonucleotide primers are decided for the amplification of the H chain V regions of the antibody MABL-1 and the antibody MABL-2.

**[0032]**

In embodiments of the invention, the 5'-end primers which contain a sequence "GANTC" providing the restriction enzyme Hinf I recognition site at the neighborhood of 5'-terminal thereof are used and the 3'-end primers which contain a nucleotide sequence "CCCGGG" providing the XmaI recognition site at the neighborhood of 5'-terminal thereof are used. Other restriction enzyme recognition site may be used instead of these sites as long as they are used for subcloning a desired DNA fragment into a cloning vector.

**[0033]**

Specifically designed PCR primers are employed to provide suitable nucleotide sequences at 5'-end and 3'-end of the cDNAs encoding the V regions of the antibodies MABL-1 and MABL-2 so that the cDNAs are readily inserted into an expression vector and appropriately function in the expression vector (e.g. this invention devises to increase transcription efficiency by inserting Kozak sequence). The

V regions of the antibodies MABL-1 and MABL-2 obtained by amplifying by PCR using these primers are inserted into HEF expression vector containing the desired human C region (see WO92/19759). The cloned DNAs can be sequenced by using any conventional process which comprises, for example, inserting the DNAs into a suitable vector and then sequencing using the automatic DNA sequencer (Applied Biosystems).

**[0034]**

Each V region of the reconstructed polypeptide of the present invention can be humanized by using conventional techniques (e.g. Sato, K. et al., Cancer Res., 53, 1-6 (1993)). Once a DNA encoding a humanized Fv is prepared, a humanized single chain Fv, a fragment of the humanized single chain Fv, a humanized monoclonal antibody and a fragment of the humanized monoclonal antibody can readily be produced according to conventional methods. Preferably, amino acid sequences of the V regions thereof may be partially modified, if necessary.

**[0035]**

Furthermore, a DNA derived from other mammalian origin, for example a DNA of human, can be produced in the same manner as used to produce DNA encoding the H chain V region and the L chain V region derived from mouse mentioned in the above. The resulting DNA can be used to prepare an H chain V region and an L chain V region of other mammal, especially human origin, a single chain Fv derived from

human and a fragment thereof, and a monoclonal antibody of human origin and a fragment thereof.

**[0036]**

As mentioned above, when the aimed DNAs encoding the V regions of the reconstructed polypeptides and the V regions of the humanized reconstructed polypeptides are prepared, the expression vectors containing them and hosts transformed with the vectors can be obtained according to conventional methods. Further, the hosts can be cultured according to a conventional method to produce the reconstructed single chain Fv, the reconstructed humanized single chain Fv, the humanized monoclonal antibodies and fragments thereof. They can be isolated from cells or a medium and can be purified into a homogeneous mass. For this purpose any isolation and purification methods conventionally used for proteins, e.g. chromatography, ultra-filtration, salting-out and dialysis, may be employed in combination, if necessary, without limitation thereto.

**[0037]**

For the production of the reconstructed polypeptides binding to cells with human IAP of the present invention, any expression systems can be employed, for example, eukaryotic cells such as animal cells, e.g., established mammalian cell lines, filamentous fungi and yeast, and prokaryotic cells such as bacterial cells e.g., E. coli. Preferably, the reconstructed polypeptides of the

invention are expressed in mammalian cells, for example COS7 cells or CHO cells.

**[0038]**

In these cases, conventional promoters useful for the expression in mammalian cells can be used. Preferably, 5 human cytomegalovirus (HCMV) immediate early promoter is used. Expression vectors containing the HCMV promoter include HCMV-VH-HC $\gamma$  1, HCMV-VL-HCK and the like which are derived from pSV2neo (WO92/19759).

**[0039]**

Additionally, other promoters for gene expression in mammal cell which may be used in the invention include virus promoters derived form retrovirus, polyoma virus, adenovirus and simian virus 40 (SV40) and promoters derived 15 from mammal such as human polypeptide-chain elongation factor-1 $\alpha$  (HEF-1 $\alpha$ ). SV40 promoter can easily be used according to the method of Mulligan, R.C., et al. (Nature 277, 108-114 (1979)) and HEF-1 $\alpha$  promoter can also be used according to the methods of Mizushima, S. et al. (Nucleic 20 Acids Research, 18, 5322 (1990)).

**[0040]**

Replication origin (ori) which can be used in the invention includes ori derived from SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV) and the like. An 25 expression vector may contain, as a selection marker, phosphotransferase APH (3') II or I (neo) gene, thymidine kinase (TK) gene, E. coli xanthine-guanine phosphoribosyl

transferase (Ecogpt) gene or dihydrofolate reductase (DHFR) gene.

**[0041]**

The antigen-binding activity of the reconstructed polypeptide as prepared above can be evaluated using the binding-inhibitory ability of the mouse antibodies, MABL-1 and MABL-2, to human IAP as an index. Concretely, the activity is evaluated in terms of the absence or presence of concentration-dependent inhibition of the binding of said monoclonal antibody as an index.

**[0042]**

More in detail, animal cells transformed with an expression vector containing a DNA encoding the reconstructed polypeptide of the invention, e.g., COS7 cells or CHO cells, are cultured. The cultured cells and/or the supernatant of the medium or the reconstructed polypeptide purified from them are used to determine the binding to antigen. As a control is used a supernatant of the culture medium in which cells transformed only with the expression vector were cultured. A test sample of the reconstructed polypeptide of the invention or the supernatant of the control is added to mouse leukemia cell line, L1210 cells, expressing human IAP and then an assay such as the flow cytometry is carried out to evaluate the antigen-binding activity.

**[0043]**

In vitro evaluation of apoptosis-inducing effect is performed in the following manner: A test sample of the above reconstructed polypeptide is added to the cells which are expressing the antibody or cells into which the gene for the antibody has been introduced, and is evaluated by whether cell death is induced in a manner specific to the human IAP-antigen.

**[0044]**

In vivo evaluation of the apoptosis-inducing effect is carried out in the following manner: A mouse model of human myeloma is prepared. To the mice is intravenously administered the monoclonal antibody or the reconstructed polypeptide of the invention, which induces apoptosis of nucleated blood cells having IAP. To mice of a control group is administered PBS alone. The induction of apoptosis is evaluated in terms of antitumor effect based on the change of human IgG content in serum of the mice and their survival time.

**[0045]**

Hemagglutination effect is tested in the following manner: A suspension of erythrocytes is prepared from blood of healthy donors. Test samples of different concentrations are added to the suspension, which are then incubated to determine the hemagglutination.

**[0046]**

The reconstructed polypeptides of the invention, which comprises two or more H chain V regions and two or

more L chain V regions may be a dimer, trimer or tetramer of the single-chain Fv comprising one H chain V region and one L chain V region, or a polypeptide in which two or more H chain V regions and two or more L chain V regions are connected. It is considered that owing to such construction the peptide mimics three dimensional structure of the antigen binding site of the parent monoclonal antibody and therefore retains an excellent antigen-binding property.

**[0047]**

The reconstructed polypeptide of the invention has a superior mobility to tissues or tumors over whole IgG and a remarkably reduced or no side effect of hemagglutination. Therefore, it is expected that the peptide of the invention can be used as a therapeutic agent for blood diseases, for example, leukemia such as acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, adult T-cell leukemia, multiple myeloma, mixed leukemia and hairy cell leukemia, malignant lymphoma (Hodgkin's disease, non-Hodgkin's lymphoma), hypoplastic anemia, osteomyelodysplasia and polycythemia vera. It is further expected that the peptide of the invention can be used as a contrast agent by RI-labeling. The effect of the peptide can be enhanced by attaching to a RI-compound or a toxin.

**[0048]**

The present invention will concretely be illustrated in reference to the following examples, which in no way limit the scope of the invention.

**[0049]**

5      **[EXAMPLES]**

For illustrating the production process of the reconstructed polypeptides of the invention, examples of producing single chain Fvs are shown below. Mouse antibodies against human IAP, MABL-1 and MABL-2 were used in the 10 examples of producing the reconstructed polypeptides.

Hybridomas MABL-1 and MABL-2 producing them respectively were internationally deposited as FERM BP-6100 and FERM BP-6101 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, 15 Minister of International Trade and Industry (1-3 Higasi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan), an authorized depository for microorganisms, on September 11, 1997.

**[0050]**

Example 1 (Cloning of DNAs encoding V region of mouse 20 monoclonal antibodies to human IAP)

DNAs encoding variable regions of the mouse monoclonal antibodies to human IAP, MABL-1 and MABL-2, were cloned as follows.

**[0051]**

25      1.1 Preparation of messenger RNA (mRNA)

mRNAs of the hybridomas MABL-1 and MABL-2 were obtained by using mRNA Purification Kit (Pharmacia Biotech).

[0052]

1.2 Synthesis of double-stranded cDNA

Double-stranded cDNA was synthesized from about 1 µg of the mRNA using Marathon cDNA Amplification Kit (CLONTECH) and an adapter was linked thereto.

5

[0053]

1.3 PCR Amplification of genes encoding variable regions of an antibody by

PCR was carried out using Thermal Cycler (PERKIN ELMER).

10

[0054]

(1) Amplification of a gene coding for L chain V region of MABL-1

Primers used for the PCR method are Adapter Primer-1 (CLONTECH) shown in SEQ ID No. 1, which hybridizes to a partial sequence of the adapter, and MKC (Mouse Kappa Constant) primer (Bio/Technology, 9, 88-89, 1991) shown in SEQ ID No. 2, which hybridizes to the mouse kappa type L chain V region.

15

[0055]

50 µl of the PCR solution contains 5 µl of 10 × PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 units of a DNA polymerase, AmpliTaq Gold (PERKIN ELMER), 0.2 µM of the adapter primer of SEQ ID No. 1, 0.2 µM of the MKC primer of SEQ ID No. 2 and 0.1 µg of the double-stranded cDNA derived from MABL-1. The solution was preheated at 94°C of the initial temperature for 9

25

minutes and then heated at 94°C for 1 minute, at 60°C for 1 minute and at 72°C for 1 minute 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 10 minutes.

5 [0056]

(2) Amplification of cDNA encoding H chain V region of MABL-1

10 The Adapter Primer-1 shown in SEQ ID No. 1 and MHC- $\gamma$ 1 (Mouse Heavy Constant) primer (Bio/Technology, 9, 88-89, 1991) shown in SEQ ID No. 3 were used as primers for PCR.

[0057]

15 The amplification of cDNA was performed according to the method of the amplification of the L chain V region gene, which was described in Example 1.3-(1), except for using 0.2  $\mu$ M of the MHC- $\gamma$ 1 primer instead of 0.2  $\mu$ M of the MKC primer.

[0058]

20 (3) Amplification of cDNA encoding L chain V region of MABL-2

The Adapter Primer-1 of SEQ ID No. 1 and the MKC primer of SEQ ID No. 2 were used as primers for PCR.

[0059]

25 The amplification of cDNA was carried out according to the method of the amplification of the L chain V region gene of MABL-1 which was described in Example 1.3-(1), except for using 0.1  $\mu$ g of the double-stranded cDNA

derived from MABL-2 instead of 0.1 µg of the double-stranded cDNA from MABL-1.

**[0060]**

(4) Amplification of cDNA encoding H chain V region of  
5 MABL-2

The Adapter Primer-1 of SEQ ID No. 1 and MHC-γ2a primer (Bio/Technology, 9, 88-89, 1991) shown in SEQ ID No. 4 were used as primers for PCR.

**[0061]**

10 The amplification of cDNA was performed according to the method of the amplification of the L chain V region gene, which was described in Example 1.3-(3), except for using 0.2 µM of the MHC-γ2a primer instead of 0.2 µM of the MKC primer.

15 **[0062]**

1.4 Purification of PCR products

The DNA fragment amplified by PCR as described above was purified using the QIAquick PCR Purification Kit (QIAGEN) and dissolved in 10 mM Tris-HCl (pH 8.0) containing 20 1 mM EDTA.

**[0063]**

1.5 Ligation and Transformation

About 140 ng of the DNA fragment comprising the gene encoding the mouse kappa type L chain V region derived from MABL-1 as prepared above was ligated with 50 ng of pGEM-T Easy vector (Promega) in the reaction buffer comprising 30 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM

dithiothreitol, 1 mM ATP and 3 units of T4 DNA Ligase (Promega) at 15°C for 3 hours.

**[0064]**

Then, 1 µl of the reaction mixture was added to 50  
5 µl of E. coli DH5α competent cells (Toyobo Inc.) and the  
cells were stored on ice for 30 minutes, incubated at 42°C  
for 1 minute and stored on ice for 2 minutes again. 100 µl  
of SOC medium (GIBCO BRL) was added. The cells of E. coli  
were plated on LB (Molecular Cloning: A Laboratory Manual,  
10 Sambrook et al., Cold Spring Harbor Laboratory Press, 1989)  
agar medium containing 100 µg/ml of ampicillin (SIGMA) and  
cultured at 37°C overnight to obtain the transformant of E. coli.

**[0065]**

15 The transformant was cultured in 3 ml of LB medium  
containing 50 µg/ml of ampicillin at 37°C overnight and the  
plasmid DNA was prepared from the culture using the QIAprep  
Spin Miniprep Kit (QIAGEN).

**[0066]**

20 The resulting plasmid comprising the gene encoding  
the mouse kappa type L chain V region derived from the  
hybridoma MABL-1 was designated as pGEM-M1L.

**[0067]**

According to the same manner as described above, a  
25 plasmid comprising the gene encoding the mouse H chain V  
region derived from the hybridoma MABL-1 was prepared from  
the purified DNA fragment and designated as pGEM-M1H.

**[0068]**

A plasmid comprising the gene encoding the mouse kappa type L chain V region derived from the hybridoma MABL-2 was prepared from the purified DNA fragment and designated 5 as pGEM-M2L.

**[0069]**

A plasmid comprising the gene encoding the mouse H chain V region derived from the hybridoma MABL-2 was prepared from the purified DNA fragment and designated as 10 pGEM-M2H.

**[0070]**

Example 2 (DNA Sequencing)

The nucleotide sequence of the cDNA encoding 15 region in the aforementioned plasmids was determined using Auto DNA Sequencer (Applied Biosystem) and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem) according to the manufacturer's protocol.

**[0071]**

The nucleotide sequence of the gene encoding the L 20 chain V region from the mouse antibody MABL-1, which is included in the plasmid pGEM-M1L, is shown in SEQ ID No. 5.

**[0072]**

The nucleotide sequence of the gene encoding the H 25 chain V region from the mouse antibody MABL-1, which is included in the plasmid pGEM-M1H, is shown in SEQ ID No. 6.

**[0073]**

The nucleotide sequence of the gene encoding the L chain V region from the mouse antibody MABL-2, which is included in the plasmid pGEM-M2L, is shown in SEQ ID No. 7.

**[0074]**

5 The nucleotide sequence of the gene encoding the H chain V region from the mouse antibody MABL-2, which is included in the plasmid pGEM-M2H, is shown in SEQ ID No. 8.

**[0075]**

Example 3 (Determination of CDR)

10 The V regions of L chain and H chain generally have a similarity in their structures and each four framework regions therein are linked by three hypervariable regions, i.e., complementarity determining regions (CDR). An amino acid sequence of the framework is relatively well 15 conserved, while an amino acid sequence of CDR has extremely high variation (Kabat, E.A., et al., "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services, 1983).

**[0076]**

20 On the basis of these facts, the amino acid sequences of the variable regions from the mouse monoclonal antibodies to human IAP were applied to the database of amino acid sequences of the antibodies made by Kabat et al. to investigate the homology. The CDR regions were determined 25 based on the homology as shown in Table 1.

**[0077]**

**[Table 1]**

Table 1

<u>Plasmid</u>	<u>SEQ ID No.</u>	<u>CDR(1)</u>	<u>CDR(2)</u>	<u>CDR(3)</u>
	pGEM-M1L	5	43-58	74-80
	pGEM-M1H	6	50-54	69-85
5	pGEM-M2L	7	43-58	74-80
	pGEM-M2H	8	50-54	69-85
				113-121
				118-125
				113-121
				118-125

[0078]

Example 4 (Identification of Cloned cDNA Expression  
(Preparation of Chimera MABL-1 antibody and Chimera MABL-2  
10 antibody.)

4.1 Preparation of vectors expressing chimera MABL-1  
antibody

CDNA clones, pGEM-M1L and pGEM-M1H, encoding the V  
regions of the L chain and the H chain of the mouse antibody  
15 MABL-1, respectively, were modified by the PCR method and  
introduced into the HEF expression vector (WO92/19759) to  
prepare vectors expressing chimera MABL-1 antibody.

[0079]

A forward primer MLS (SEQ ID No. 9) for the L  
20 chain V region and a forward primer MHS (SEQ ID No. 10) for  
the H chain V region were designed to hybridize to a DNA  
encoding the beginning of the leader sequence of each V  
region and to contain the Kozak consensus sequence (J. Mol.  
Biol., 196, 947-950, 1987) and HindIII restriction enzyme  
25 site. A reverse primer MLAS (SEQ ID No. 11) for the L chain  
V region and a reverse primer MHAS (SEQ ID No. 12) for the H  
chain V region were designed to hybridize to a DNA encoding

the end of the J region and to contain the splice donor sequence and BamHI restriction enzyme site.

**[0080]**

100 µl of a PCR solution comprising 10 µl of 10 × 5 PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs (dATP, dGTP, dCTP and dTTP), 5 units of DNA polymerase AmpliTaq Gold, 0.4 µM each of primers and 8 ng of the template DNA (pGEM-M1L or pGEM-M1H) was preheated at 94°C of the initial temperature for 9 minutes and then heated at 94°C for 1 minute, at 60°C 10 for 1 minute and at 72°C for 1 minute 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 10 minutes.

**[0081]**

The PCR product was purified using the QIAquick 15 PCR Purification Kit (QIAGEN) and then digested with HindIII and BamHI. The product from the L chain V region was cloned into the HEF expression vector, HEF-κ and the product from the H chain V region was cloned into the HEF expression vector, HEF-γ. After DNA sequencing, plasmids containing a 20 DNA fragment with a correct DNA sequence are designated as HEF-M1L and HEF-M1H, respectively.

**[0082]**

4.2 Preparation of vectors expressing chimera MABL-2 antibodies

25 Modification and cloning of cDNA were performed in the same manner described in Example 4.1 except for using pGEM-M2L and pGEM-M2H as template DNA instead of pGEM-M1L

and pGEM-M1H. After DNA sequencing, plasmids containing DNA fragments with correct DNA sequences are designated as HEF-M2L and HEF-M2H, respectively.

**[0083]**

5        4.3 Transfection to COS7 cells

The aforementioned expression vectors were tested in COS7 cells to observe the transient expression of the chimera MABL-1 and MABL-2 antibodies.

**[0084]**

10      (1) Transfection with genes for the chimera MABL-1 antibody

COS7 cells were co-transformed with the HEF-M1L and HEF-M1H vectors by electroporation using the Gene Pulser apparatus (BioRad). Each DNA (10 µg) and 0.8 ml of PBS with  $1 \times 10^7$  cells/ml were added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25 µF of electric capacity.

**[0085]**

20      After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into DMEM culture medium (GIBCO BRL) containing 10%  $\gamma$ -globulin-free fetal bovine serum. After culturing for 72 hours, the supernatant was collected, centrifuged to remove cell fragments and recovered.

**[0086]**

25      (2) Transfection with genes coding for the chimera MABL-2 antibody

The co-transfection to COS7 cells with the genes coding for the chimera MABL-2 antibody was carried out in

the same manner as described in Example 4.3-(1) except for using the HEF-M2L and HEF-M2H vectors instead of the HEF-M1L and HEF-M1H vectors. The supernatant was recovered in the same manner.

5 [0087]

4.4 Flow cytometry

Flow cytometry was performed using the aforementioned culture supernatant of COS7 cells to measure binding to the antigen. The culture supernatant of the COS7 10 cells expressing the chimera MABL-1 antibody or the COS7 cells expressing the chimera MABL-2 antibody, or human IgG antibody (SIGMA) as a control was added to  $4 \times 10^5$  cells of mouse leukemia cell line L1210 expressing human IAP and 15 incubated on ice. After washing, the FITC-labeled anti-human IgG antibody (Cappel) was added thereto. After incubating and washing, the fluorescence intensity thereof was measured using the FACScan apparatus (BECTON DICKINSON).

[0088]

Since the chimera MABL-1 and MABL-2 antibodies 20 were specifically bound to L1210 cells expressing human IAP, it is confirmed that these chimera antibodies have proper structures of the V regions of the mouse monoclonal antibodies MABL-1 and MABL-2, respectively (Figures 1-3).

[0089]

25 Example 5 (Preparation of reconstructed Single chain Fv (scFv) of the antibody MABL-1 and antibody MABL-2)

5.1 Preparation of reconstructed antibody MABL-1 and  
reconstructed single chain Fv (scFv) region of antibody  
MABL-2

The reconstructed single chain Fv of antibody  
5 MABL-1 was prepared as follows. The H chain V region and the  
L chain V of antibody MABL-1, and a linker were respectively  
amplified by the PCR method and were connected to produce  
the reconstructed single chain Fv of antibody MABL-1. The  
production method is illustrated in Figure 4. Six primers  
10 (A-F) were employed for the production of the single chain  
Fv of antibody MABL-1. Primers A, C and E have a sense  
sequence and primers B, D and F have an antisense sequence.

**[0090]**

The forward primer VHS for the H chain V region  
15 (Primer A, SEQ ID No. 13) was designed to hybridize to a DNA  
encoding the N-terminal of the H chain V region and to  
contain NcoI restriction enzyme recognition site. The  
reverse primer VHAS for H chain V region (Primer B, SEQ ID  
No. 14) was designed to hybridize to a DNA coding the C-  
20 terminal of the H chain V region and to overlap with the  
linker.

**[0091]**

The forward primer LS for the linker (Primer C,  
SEQ ID No. 15) was designed to hybridize to a DNA encoding  
25 the N-terminal of the linker and to overlap with a DNA  
encoding the C-terminal of the H chain V region. The reverse  
primer LAS for the linker (Primer D, SEQ ID No. 16) was

designed to hybridize to a DNA encoding the C-terminal of the linker and to overlap with a DNA encoding the N-terminal of the L chain V region.

**[0092]**

5           The forward primer VLS for the L chain V region (Primer E, SEQ ID No. 17) was designed to hybridize to a DNA encoding the C-terminal of the linker and to overlap with a DNA encoding the N-terminal of the L chain V region. The reverse primer VLAS-FLAG for L chain V region (Primer F, SEQ  
10          ID No. 18) was designed to hybridize to a DNA encoding the C-terminal of the L chain V region and to have a sequence encoding the FLAG peptide (Hopp. T. P. et al., Bio/Technology, 6, 1204-1210, 1988), two stop codons and EcoRI restriction enzyme recognition site.

15          **[0093]**

          In the first PCR step, three reactions, A-B, C-D and E-F, were carried out and PCR products thereof were purified. Three PCR products obtained from the first PCR step were assembled by their complementarity. Then, the primers A and F were added and the full length DNA encoding the reconstructed single chain Fv of antibody MABL-1 was amplified (Second PCR). In the first PCR, the plasmid pGEM-M1H encoding the H chain V region of antibody MABL-1 (see Example 2), a plasmid pSC-DP1 which comprises a DNA sequence encoding a linker region comprising: Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser (SEQ ID No. 19) (Huston, J.S., et al., Proc. Natl. Acad. Sci. USA, 85, 5879-5883,

1988) and the plasmid pGEM-M1L encoding the L chain V region of antibody MABL-1 (see Example 2) were employed as template, respectively.

**[0094]**

5               50 µl of the solution for the first PCR step comprises 5 µl of 10 × PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs, 2.5 units of DNA polymerase, AmpliTaq Gold (PERKIN ELMER), 0.4 µM each of primers and 5 ng each of template DNA. The PCR solution was preheated at 94°C of the initial  
10 temperature for 9 minutes and then heated at 94°C for 1 minute, at 65°C for 1 minute and at 72°C for 1 minute and 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 7 minutes.

15               **[0095]**

The PCR products A-B (371bp), C-D (63bp) and E-F (384bp) were purified using the QIAquick PCR Purification Kit (QIAGEN) and were assembled in the second PCR. In the second PCR, 98 µl of a PCR solution comprising 120 ng of the first PCR product A-B, 20 ng of the PCR product C-D and 120 ng of the PCR product E-F, 10 µl of 10 × PCR Buffer II, 2mM MgCl<sub>2</sub>, 0.16 mM dNTPs, 5 units of DNA polymerase AmpliTaq Gold (PERKIN ELMER) was preheated at 94°C of the initial temperature for 8 minutes and then heated at 94°C for 2 minutes, at 65°C for 2 minutes and at 72°C for 2 minutes in order. This temperature cycle was repeated twice and then 25 0.4 µM each of primers A and F were added into the reaction,

respectively. The mixture was preheated at 94°C of the initial temperature for 1 minutes and then heated at 94°C for 1 minute, at 65°C for 1 minute and at 72°C for 1 minute and 20 seconds in order. This temperature cycle was repeated  
5 35 times and then the reaction mixture was further heated at 72°C for 7 minutes.

**[0096]**

A DNA fragment of 843 bp produced by the second PCR was purified and digested by NcoI and EcoRI. The  
10 resultant DNA fragment was cloned into pSCFVT7 vector. The expression vector pSCFVT7 contains a pelB signal sequence suitable for E. coli periplasmic expression system (Lei, S.P., et al., J. Bacteriology, 169, 4379-4383, 1987). After the DNA sequencing, the plasmid containing the DNA fragment encoding correct amino acid sequence of the reconstructed single chain Fv of antibody MABL-1 is designated as "pscM1" (see Figure 5). The nucleotide sequence and the amino acid sequence of the reconstructed single chain Fv of antibody MABL-1 contained in the plasmid pscM1 are shown in SEQ ID  
15 No. 20.  
20

**[0097]**

The pscM1 vector was modified by the PCR method to prepare a vector expressing the reconstructed single chain Fv of antibody MABL-1 in mammalian cells. The resultant DNA  
25 fragment was introduced into pCHO1 expression vector. This expression vector, pCHO1, was constructed by digesting DHFR-  
ΔE-rvH-PM1-f (WO92/19759) with EcoRI and SmaI to eliminate

the antibody gene and connecting the EcoRI-NotI-BamHI Adapter (Takara Shuzo) thereto.

**[0098]**

As a forward primer for PCR, Sal-VHS primer shown in SEQ ID No. 21 was designed to hybridize to a DNA encoding the N-terminal of the H chain V region and to contain SalI restriction enzyme recognition site. As a reverse primer for PCR, FRHlanti primer shown in SEQ ID No. 22 was designed to hybridize to a DNA encoding the end of the first framework sequence.

**[0099]**

100 µl of PCR solution comprising 10 µl of 10 × PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs, 5 units of the DNA polymerase, AmpliTaq Gold, 0.4 µl M each of primer and 8 ng of the template DNA (pscM1) was preheated at 95°C of the initial temperature for 9 minutes and then heated at 95°C for 1 minute, at 60°C for 1 minute and at 72°C for 1 minute and 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 7 minutes.

**[0100]**

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and digested by SalI and MboII to obtain a DNA fragment encoding the N-terminal of the reconstructed single chain Fv of antibody MABL-1. The pscM1 vector was digested by MboII and EcoRI to obtain a DNA fragment encoding the C-terminal of the reconstructed single

chain Fv of antibody MABL-1. The SalI-MboII DNA fragment and  
the MboII-EcoRI DNA fragment were cloned into pCHO1-Igs  
vector. After DNA sequencing, the plasmid comprising the  
desired DNA sequence was designated as "pCHOM1" (see Figure  
5 6). The expression vector, pCHO1-Igs, contains a mouse IgG1  
signal sequence suitable for the secretion-expression system  
in mammalian cells (Nature, 322, 323-327, 1988). The  
nucleotide sequence and the amino acid sequence of the  
reconstructed single chain Fv of antibody MABL-1 contained  
10 in the plasmid pCHOM1 are shown in SEQ ID No. 23.

**[0101]**

5.2 Preparation of reconstructed single chain Fv of  
antibody MABL-2

The reconstructed single chain Fv of antibody  
15 MABL-2 was prepared in accordance with the aforementioned  
Example 5.1. Employed in the first PCR step were plasmid  
pGEM-M2H encoding the H chain V region of MABL-2 (see  
Example 2) instead of pGEM-M1H and plasmid pGEM-M2L encoding  
the L chain V region of MABL-2 (see Example 2) instead of  
pGEM-M1L, to obtain a plasmid pscM2 which comprises a DNA  
20 fragment encoding the desired amino acid sequence of the  
single chain Fv of antibody MABL-2. The nucleotide sequence  
and the amino acid sequence of the reconstructed single  
chain Fv of antibody MABL-2 contained in the plasmid pscM2  
25 are shown in SEQ ID No. 24.

**[0102]**

The pscM2 vector was modified by the PCR method to prepare a vector, pCHOM2, for the expression in mammalian cells which contains the DNA fragment encoding the correct amino acid sequence of reconstructed the single chain Fv of antibody MABL-2. The nucleotide sequence and the amino acid sequence of the reconstructed single chain Fv of antibody MABL-2 contained in the plasmid pCHOM2 are shown in SEQ ID No. 25.

**[0103]**

10       5.3 Transfection to COS7 cells

The pCHOM2 vector was tested in COS7 cells to observe the transient expression of the reconstructed single chain Fv of antibody MABL-2.

**[0104]**

15       The COS7 cells were transformed with the pCHOM2 vector by electroporation using the Gene Pulser apparatus (BioRad). The DNA (10 µg) and 0.8 ml of PBS with  $1 \times 10^7$  cells/ml were added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25 µF of electric capacity.

20       **[0105]**

After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into IMDM culture medium (GIBCO BRL) containing 10% fetal bovine serum. After culturing for 72 hours, the supernatant was collected, centrifuged to remove cell fragments and recovered.

25       **[0106]**

5.4 Detection of the reconstructed single chain Fv of antibody MABL-2 in culture supernatant of COS7 cells

The existence of the single chain Fv of antibody MABL-2 in the culture supernatant of COS7 cells which had been transfected with the pCHOM2 vector was confirmed by the Western Blotting method.

**[0107]**

The culture supernatant of COS7 cells transfected with the pCHOM2 vector and the culture supernatant of COS7 cells transfected with the pCHO1 as a control were subjected to SDS electrophoresis and transferred to REINFORCED NC membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk (Morinaga Nyu-gyo), washed with 0.05% Tween 20-PBS and mixed with an anti-FLAG antibody (SIGMA). The membrane was incubated at room temperature, washed and mixed with alkaline phosphatase-conjugated mouse IgG antibody (Zymed). After incubating and washing at room temperature, the substrate solution (Kirkegaard Perry Laboratories) was added to develop color (Figure 7).

**[0108]**

A FLAG-peptide-specific protein was detected only in the culture supernatant of the pCHOM2 vector-introduced COS7 cells and thus it is confirmed that the reconstructed single chain Fv of antibody MABL-2 was secreted in this culture supernatant.

**[0109]**

5.5 Flow cytometry

Flow cytometry was performed using the aforementioned COS7 cells culture supernatant to measure the binding to the antigen. The culture supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 or the culture supernatant of COS7 cells transformed with pCHO1 vector as a control was added to  $2 \times 10^5$  cells of the mouse leukemia cell line L1210 expressing human Integrin Associated Protein (IAP) or the cell line L1210 transformed with pCOS1 as a control. After incubating on ice and washing, the mouse anti-FLAG antibody (SIGMA) was added. Then the cells were incubated and washed. Then, the FITC labeled anti-mouse IgG antibody (BECTON DICKINSON) was added thereto and the cells were incubated and washed again. Subsequently, the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON).

**[0110]**

Since the single chain Fv of antibody MABL-2 was specifically bound to L1210 cells expressing human IAP, it is confirmed that the reconstructed single chain Fv of antibody MABL-2 has an affinity to human Integrin Associated Protein (IAP) (see Figures 8-11).

**[0111]**

5.6 Competitive ELISA

The binding activity of the reconstructed single chain Fv of antibody MABL-2 was measured based on the inhibiting activity against the binding of mouse monoclonal antibodies to the antigen.

[0112]

The anti-FLAG antibody adjusted to 1 µg/ml was added to each well on 96-well plate and incubated at 37°C for 2 hours. After washing, blocking was performed with 1% BSA-PBS. After incubating and washing at a room temperature, the culture supernatant of COS7 cells into which the secretion-type human IAP antigen gene (SEQ ID No. 26) had been introduced was diluted with PBS into twofold volume and added to each well. After incubating and washing at a room temperature, a mixture of 50 µl of the biotinized MABL-2 antibody adjusted to 100 ng/ml and 50 µl of sequentially diluted supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 were added into each well. After incubating and washing at a room temperature, the alkaline phosphatase-conjugated streptoavidin (Zymed) was added into each well. After incubating and washing at a room temperature, the substrate solution (SIGMA) was added and absorbance of the reaction mixture in each well was measured at 405 nm.

20 [0113]

The results revealed that the reconstructed single chain Fv of antibody MABL-2 (MABL2-scFv) evidently inhibited concentration-dependently the binding of the mouse antibody MABL-2 to human IAP antigen in comparison with the culture supernatant of the PCHO1-introduced COS7 cells as a control (Figure 12). Accordingly, it is suggested that the reconstructed single chain Fv of antibody MABL-2 has the

correct structure of each of the V regions from the mouse monoclonal antibody MABL-2.

**[0114]**

5.7 Apoptosis-inducing Effect in vitro

5 An apoptosis-inducing action of the reconstructed single chain Fv of antibody MABL-2 was examined by Annexin-V staining (Boehringer Mannheim) using the L1210 cells transfected with human IAP gene, the L1210 cells transfected with the pCOS1 vector as a control and CCRF-CEM cells.

10 **[0115]**

To each  $1 \times 10^5$  cells of the above cells was added the culture supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 or the culture supernatant of COS7 cells transfected with the pCHO1 vector as a control at 50% final concentration and the mixtures were cultured for 24 hours. Then, the Annexin-V staining was performed and the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON).

15 **[0116]**

20 Results of the Annexin-V staining are shown in Figures 13-18, respectively. Dots in the left-lower region represent living cells and dots in the right-lower region represent cells at the early stage of apoptosis and dots in the right-upper region represent cells at the late stage of apoptosis. The results show that the reconstructed single 25 chain Fv of antibody MABL-2 (MABL2-scFv) remarkably induced cell death of L1210 cells specific to human IAP antigen

(Figures 13-16) and that the reconstructed single chain Fv also induced remarkable cell death of CCRF-CEM cells in comparison with the control (Figures 17-18).

**[0117]**

5       5.8 Expression of MABL-2 derived single chain Fv in CHO  
      cells

CHO cells were transfected with the pCHOM2 vector to establish a CHO cell line which constantly expresses the single chain Fv (polypeptide) derived from the antibody  
10       MABL-2.

CHO cells were transformed with the pCHOM2 vector by the electroporation using the Gene Pulser apparatus (BioRad). A mixture of DNA (10 µg) and 0.7 ml of PBS with CHO cells ( $1 \times 10^7$  cells/ml) was added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25 µF of electric capacity. After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into nucleic acid free α-MEM medium (GIBCO BRL) containing 10% fetal bovine serum and cultured. The expression of desired protein in the resultant clones was confirmed by SDS-PAGE and a clone with a high expression level was selected as a cell line producing the single chain Fv derived from the antibody MABL-2. The cell line was cultured in serum-free medium CHO-S-SFM II (GIBCO BRL) containing 10 nM methotrexate (SIGMA). Then, the culture supernatant was collected, centrifuged to remove cell fragments and recovered.  
15  
20  
25

[0118]

5.9 Purification of MABL-2 derived single chain Fv produced in CHO cells

5       The culture supernatant of the CHO cell line expressing the single chain Fv obtained in Example 5.8 was concentrated up to twenty times using a cartridge for the artificial dialysis (PAN130SF, ASAHI MEDICALS). The concentrated solution was stored at -20°C and thawed on purification.

10      Purification of the single chain Fv from the culture supernatant of the CHO cells was performed using three kinds of chromatography, i.e., Blue-sepharose, a hydroxyapatite and a gel filtration.

[0119]

15      (1) Blue-sepharose column chromatography

The concentrated supernatant was diluted to ten times with 20 mM acetate buffer (pH 6.0) and centrifuged to remove insoluble materials ( $10000 \times \text{ rpm}$ , 30 minutes). The supernatant was applied onto a Blue-sepharose column (20 ml) equilibrated with the same buffer. After washing the column with the same buffer, proteins adsorbed in the column were eluted by a stepwise gradient of NaCl in the same buffer, 0.1, 0.2, 0.3, 0.5 and up to 1.0 M. The pass-through fraction and each eluted fraction were analyzed by SDS-PAGE. The fractions in which the single chain Fv were confirmed (the fractions eluted at 0.1 to 0.3M NaCl) were pooled and

concentrated up to approximately 20 times using CentriPrep-10 (AMICON).

[0120]

(2) Hydroxyapatite

5           The concentrated solution obtained in (1) was diluted to 10 times with 10 mM phosphate buffer (pH 7.0) and applied onto the hydroxyapatite column (20 ml, BIORAD). The column was washed with 60 ml of 10 mM phosphate buffer (pH 7.0). Then, proteins adsorbed in the column were eluted by a  
10 linear gradient of sodium phosphate buffer up to 200 mM (see Figure 19). The analysis of each fraction by SDS-PAGE confirmed the single chain Fv in fraction A and fraction B.

[0121]

(3) Gel filtration

15           Each of fractions A and B in (2) was separately concentrated with CentriPrep-10 and applied onto TSKgel G3000SWG column (21.5 × 600 mm) equilibrated with 20 mM acetate buffer (pH 6.0) containing 0.15 M NaCl. Chromatograms are shown in Figure 20. The analysis of the  
20 fractions by SDS-PAGE confirmed that both major peaks (AI and BI) are of desired single chain Fv. In the gel filtration analysis, the fraction A was eluted at 36 kDa of apparent molecular weight and the fraction B was eluted at 76 kDa. The purified single chain Fvs (AI, BI) were analyzed with 15% SDS polyacrylamide gel. Samples were treated in the  
25 absence or presence of a reductant and the electrophoresis was carried out in accordance with the Laemmli's method.

Then the protein was stained with Coomassie Brilliant Blue. As shown in Figure 21, both AI and BI gave a single band at 35 kDa of apparent molecular weight, regardless of the absence or presence of the reductant. From the above, it is  
5 concluded that AI is a monomer of the single chain Fv and BI is a non-covalently bound dimer of the single chain Fv. The gel filtration analysis of the fractions AI and BI with TSKgel G3000SW column (7.5 × 60 mm) revealed that a peak of the monomer is detected only in the fraction AI and a peak  
10 of the dimer is detected only in the fraction BI (Figure 22).

**[0122]**

5.10 Construction of vector expressing single chain Fv derived from antibody MABL-2 in E. coli cell

15 The pscM2 vector was modified by the PCR method to prepare a vector effectively expressing the single chain Fv from the antibody MABL-2 in E. coli cells. The resultant DNA fragment was introduced into pSCFVT7 expression vector.

**[0123]**

20 As a forward primer for PCR, Nde-VHSm02 primer shown in SEQ ID No. 27 was designed to hybridize to a DNA encoding the N-terminal of the H chain V region and to contain a start codon and NdeI restriction enzyme recognition site. As a reverse primer for PCR, VLAS primer  
25 shown in SEQ ID No. 28 was designed to hybridize to a DNA encoding the C-terminal of the L chain V region and to contain two stop codons and EcoRI restriction enzyme

recognition site. The forward primer, Nde-VHSm02, comprises five point mutations in the part hybridizing to the DNA encoding the N-terminal of the H chain V region for the effective expression in E. coli.

5 [0124]

100  $\mu$ l of a PCR solution comprising 10  $\mu$ l of 10 x PCR Buffer #1, 1 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 5 units of KOD DNA polymerase (all from TOYOBO), 1  $\mu$ M of each primer and 100 ng of a template DNA (pscM2) was heated at 98°C for 15 seconds, 10 at 65°C for 2 seconds and at 74°C for 30 seconds in order. This temperature cycle was repeated 25 times.

[0125]

15 The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and digested by NdeI and EcoRI, and then the resulting DNA fragment was cloned into pSCFVT7 vector, from which pelB signal sequence had been eliminated by the digestion with NdeI and EcoRI. After DNA sequencing, the resulting plasmid comprising a DNA fragment with the desired DNA sequence is designated as "pscM2DEm02" 20 (see Figure 23). The nucleotide sequence and the amino acid sequence of the single chain Fv derived from the antibody MABL-2 contained in the plasmid pscM2DEm02 are shown in SEQ ID No. 29.

[0126]

25 5.11 Expression of single chain Fv derived from antibody MABL-2 in E. coli cells

E. coli BL21(DE3)pLysS (STRATAGENE) was transformed with pscM2DEm02 vector to obtain a strain of E. coli expressing the single chain Fv derived from antibody MABL-2. The resulting clones were examined for the expression of the desired protein using SDS-PAGE, and a clone with a high expression level was selected as a strain producing the single chain Fv derived from antibody MABL-2.

5 [0127]

10 5.12 Purification of single chain Fv derived from antibody MABL-2 produced in E.coli

A single colony of E. coli obtained by the transformation was cultured in 3 ml of LB medium at 28°C for 7 hours and then in 70 ml of LB medium at 28°C overnight. This pre-culture was transplanted to 7 L of LB medium and cultured at 28°C with stirring at 300 rpm using the Jar-fermenter. When an absorbance of the medium reached O.D.=1.5, the bacteria were induced with 1 mM IPTG and then cultured for 3 hours.

15 [0128]

20 The culture medium was centrifuged (10000 × g, 10 minutes) and the precipitated bacteria were recovered. To the bacteria was added 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M NaCl and 1% Triton X-100 and the bacteria were disrupted by ultrasonication (out put: 4, duty cycle: 70%, 1 minute × 10 times). The suspension of disrupted bacteria was centrifuged (12000 × g, 10 minutes) to precipitate inclusion body. Isolated inclusion body was

mixed with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M NaCl and 4% Triton X-100, treated by ultrasonication (out put: 4, duty cycle: 50%, 30 seconds × 2 times) again and centrifuged (12000 × g, 10 minutes) to isolate the desired protein as precipitate and to remove 5 containment proteins included in the supernatant.

**[0129]**

The inclusion body comprising the desired protein was lysed in 50 mM Tris-HCl buffer (pH 8.0) containing 6 M Urea, 5 mM EDTA and 0.1 M NaCl and applied onto Sephadryl S-10 300 gel filtration column (5 × 90 cm, Amersham Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 4M Urea, 5 mM EDTA, 0.1 M NaCl and 10 mM mercaptoethanol at a flow rate of 5 ml/minutes to remove associated single 15 chain Fvs with high-molecular weight. The obtained fractions were analyzed with SDS-PAGE and the fractions with high purity of the protein were diluted with the buffer used in the gel filtration up to O.D<sub>280</sub>=0.25. Then, the fractions were dialyzed three times against 50 mM Tris-HCl buffer (pH 20 8.0) containing 5 mM EDTA, 0.1 M NaCl, 0.5 M Arg, 2 mM glutathione in the reduced form and 0.2 mM glutathione in the oxidized form in order for the protein to be refolded. Further, the fraction was dialyzed three times against 20 mM acetate buffer (pH 6.0) containing 0.15 M NaCl to exchange 25 the buffer.

**[0130]**

The dialysate product was applied onto Superdex  
200 pg gel filtration column (2.6 x 60 cm, Amersham  
Pharmacia) equilibrated with 20 mM acetate buffer (pH 6.0)  
containing 0.15 M NaCl to remove a small amount of high  
5 molecular weight protein which was intermolecularly  
crosslinked by S-S bonds. As shown in Figure 24, two peaks,  
major and sub peaks, were eluted after broad peaks which are  
expectedly attributed to an aggregate with a high molecular  
weight. The analysis by SDS-PAGE (see Figure 21) and the  
10 elution positions of the two peaks in the gel filtration  
analysis suggest that the major peak is of the monomer of  
the single chain Fv and the sub peak is of the non-  
covalently bound dimer of the single chain Fv.

**[0131]**

15 5.13 Apoptosis-inducing activity in vitro of single chain Fv  
derived from antibody MABL-2

An apoptosis-inducing action of the single chain  
Fv from antibody MABL-2 (MABL2-scFv) produced by the CHO  
cells and E. coli was examined according to two protocols by  
20 Annexin-V staining (Boehringer Mannheim) using the L1210  
cells (hIAP/L1210) into which human IAP gene had been  
introduced.

**[0132]**

Sample antibodies at the final concentration of 3  
25 µg/ml were added to  $5 \times 10^4$  cells of hIAP/L1210 cell line  
and cultured for 24 hours. Sample antibodies, i.e., the  
monomer and the dimer of the single chain Fv of MABL-2 from

the CHO cells obtained in Example 5.9, the monomer and the dimer of the single chain Fv of MABL-2 from E. coli obtained in Example 5.12, and the mouse IgG antibody as a control were analyzed. After culturing, the Annexin-V staining was  
5 carried out and the fluorescence intensity thereof was measured using the FACScan apparatus (BECTON DICKINSON).

**[0133]**

Results of the analysis by the Annexin-V staining are shown in Figures 25-29. The results show that the dimers  
10 of the single chain Fv polypeptide of MABL-2 produced in the CHO cells and E. coli remarkably induced cell death (Figures 26, 27) in comparison with the control (Figure 25), while no apoptosis-inducing action was observed in the monomers of the single chain Fv polypeptide of MABL-2 produced in the  
15 CHO cells and E. coli (Figures 28, 29).

**[0134]**

5.14 Antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with a model mouse of human myeloma  
(1) Quantitative measurement of human IgG in mouse serum

20 Measurement of human IgG produced by human myeloma cell and contained in mouse serum was carried out by the following ELISA. 100  $\mu$ L of goat anti-human IgG antibody (BIOSOURCE, Lot#7902) diluted to 1  $\mu$ g/mL with 0.1% bicarbonate buffer (pH 9.6) was added to each well on 96  
25 wells plate (Nunc) and incubated at 4°C overnight so that the antibody was immobilized. After blocking, 100  $\mu$ L of the stepwisely diluted mouse serum or human IgG (CAPPÉL,

Lot#00915) as a standard was added to each well and  
incubated for 2 hours at a room temperature. After washing,  
100  $\mu$ L of alkaline phosphatase-labeled anti-human IgG  
antibody (BIOSOURCE, Lot#6202) which had been diluted to  
5 5000 times was added, and incubation was carried out for 1  
hour at a room temperature. After washing, a substrate  
solution was added. After incubation, absorbance at 405 nm  
was measured using the MICROPLATE READER Model 3550  
(BioRad). The concentration of human IgG in the mouse serum  
10 was calculated based on the calibration curve obtained from  
the absorbance values of human IgG as the standard.

**[0135]**

(2) Preparation of antibodies for administration

15 The monomer and the dimer of the scFv/CHO  
polypeptide were respectively diluted to 0.4 mg/mL or 0.25  
mg/mL with sterile filtered PBS(–) on the day of  
administration to prepare samples for the administration.

**[0136]**

(3) Preparation of a mouse model of human myeloma

20 A mouse model of human myeloma was prepared as  
follows. KPMM2 cells passaged *in vivo* (JP-Appl. 7-236475) by  
SCID mouse (Japan Clare) were suspended in RPMI1640 medium  
(GIBCO-BRL) containing 10% fetal bovine serum (GIBCO-BRL)  
and adjusted to  $3 \times 10^7$  cells/mL. 200  $\mu$ L of the KPMM2 cell  
25 suspension ( $6 \times 10^6$  cells/mouse) was transplanted to the  
SCID mouse (male, 6 week-old) via caudal vein thereof, which  
had been subcutaneously injected with the asialo GM1

antibody (WAKO JUNYAKU, 1 vial dissolved in 5 mL) a day before the transplantation.

[0137]

(4) Administration of antibodies

5           The samples of the antibodies prepared in (2), the monomer (250 µL) and the dimer (400 µL), were administered to the model mice of human myeloma prepared in (3) via caudal vein thereof. The administration was started from three days after the transplantation of KPMM2 cells and was 10 carried out twice a day for three days. As a control, 200 µL of sterile filtered PBS(–) was likewise administered twice a day for three days via caudal vein. Each group consisted of seven mice.

[0138]

15           (5) Evaluation of antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with the model mouse of human myeloma

20           The antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with the model mice of human myeloma was evaluated in terms of the change of human IgG concentration in the mouse serum and survival time of the mice. The change of human IgG concentration was determined 25 by measuring it in the mouse serum collected at 24 days after the transplantation of KPMM2 cells by ELISA described in the above (1). The amount of serum human IgG (M protein) in the serum of the PBS(–)-administered group (control) increased to about 8500 µg/mL, whereas the amount of human

IgG of the scFv/CHO dimer-administered group was remarkably low, that is, as low as one-tenth or less than that of the control group. Thus, the results show that the dimer of scFv/CHO strongly inhibits the growth of the KPMM2 cells  
5 (Figure 30). As shown in Figure 31, a remarkable elongation of the survival time was observed in the scFv/CHO dimer-administered group in comparison with the PBS(-)-administered group.

**[0139]**

From the above, it is confirmed that the dimer of scFv/CHO has an antitumor effect for the human myeloma model mice. It is considered that the antitumor effect of the dimer of scFv/CHO, the reconstructed polypeptide of the invention, results from the apoptosis-inducing action of the  
15 reconstructed polypeptide.

**[0140]**

From the above examples, as shown in Figure 32, it is considered that a polypeptide of single chain which two H chain V regions and two L chain V regions are connected through a linker has an effect as well as a dimer of single  
20 chain Fv comprising an H chain V region and an L chain V region.

**[0141]**

5.15 Hemagglutination Test

Hemagglutination test and determination of hemagglutination were carried out in accordance with "Immuno-Biochemical Investigation", Zoku-Seikagaku Jikken  
25

Koza, edited by the Biochemical Society of Japan, published by Tokyo Kagaku Dojin.

Blood was taken from a healthy donor using heparin-treated syringes and washed with PBS(-) three times, and then erythrocyte suspension with a final concentration of 2% in PBS(-) was prepared. Test samples were the antibody MABL-2, the monomer and the dimer of the single chain Fv polypeptide produced by the CHO cells, and the monomer and the dimer of the single chain Fv polypeptide produced by E. coli, and the control was mouse IgG (ZYMED). For the investigation of the hemagglutination effect, round bottom 96-well plates available from Falcon were used. 50 µL per well of the aforementioned antibody samples and 50 µL of the 2% erythrocyte suspension were added and mixed in the well. After incubation for 2 hours at 37°C, the reaction mixtures were stored at 4°C overnight and the hemagglutination thereof was determined. As a control, 50 µL per well of PBS(-) was used and the hemagglutination test was carried out in the same manner. The mouse IgG and antibody MABL-2 were employed at 0.01, 0.1, 1.0, 10.0 or 100.0 µg/mL of the final concentration of the antibodies. The single chain Fvs were employed at 0.004, 0.04, 0.4, 4.0, 40.0 or 80.0 µg/mL of the final concentration and further at 160.0 µg/mL only in the case of the dimer of the polypeptide produced by E. coli. Results are shown in the Table 2. In the case of antibody MABL-2, the hemagglutination was observed at a concentration of more than 0.1 µg/mL, whereas no

hemagglutination was observed for both the monomer and the dimer of the single chain Fv.

**[0142]**

Table 2 **Hemagglutination Test**

	Control	0.01	0.1	1	10	100	μg/mL		
mIgG	-	-	-	-	-	-			
MABL-2 (intact)	-	-	+	+++	+++	++			
	Control	0.004	0.04	0.4	4	40	80	μg/mL	
scFv/CHO monomer	-	-	-	-	-	-	-		
scFv/CHO dimer	-	-	-	-	-	-	-		
	Control	0.004	0.04	0.4	4	40	80	160	μg/mL
scFv/E.coli monomer	-	-	-	-	-	-	-		
scFv/E.coli dimer	-	-	-	-	-	-	-		

**[0143]**

**[Effect of the Invention]**

As a reconstructed polypeptide of this invention is characterized by inducing apoptosis in nuclear blood cells having integrin associated protein (IAP) without causing hemagglutination, it is useful as a remedy for blood diseases, for example, leukemia such as acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, adult T-cell leukemia, multiple myeloma, mixed leukemia and hairy cell leukemia, malignant lymphoma (Hodgkin's disease, non-Hodgkin's lymphoma), hypoplastic anemia, osteomyelodysplasia and polycythemia vera.

**[0144]**

Sequence Listings

【0144】

## 【配列表】

## SEQUENCE LISTING

&lt;110&gt; CHUGAI SEIYAKU KABUSHIKI KAISHA

&lt;120&gt; A polypeptide inducible apoptosis

&lt;130&gt; DOJ-5404

&lt;160&gt; 26

&lt;210&gt; 1

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 1

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&lt;210&gt; 2

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

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&lt;211&gt; 28

&lt;212&gt; DNA

整理番号=D O J - 5 4 0 4

提出日 平成12年 4月17日  
特願2000-115246 頁： 41/ 68

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5 10 15

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整理番号=D O J - 5 4 0 4

提出日 平成12年 4月17日  
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Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser		
35	40	45
cag agc ctt cta cac agt aaa gga aac acc tat tta caa tgg tac		180
Gln Ser Leu Leu His Ser Lys Gly Asn Thr Tyr Leu Gln Trp Tyr		
50	55	60
cta cag aag cca ggc cag tct cca aag ctc ctg atc tac aaa gtt		225
Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val		
65	70	75
tcc aac cga ttt tct ggg gtc cca gac agg ttc agt ggc agt gga		270
Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly		
80	85	90
tca ggg aca gat ttc aca ctc aag atc agc aga gtg gag gct gag		315
Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu		
95	100	105
gat ctg gga gtt tat ttc tgc tct caa agt aca cat gtt ccg tac		360
Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr		
110	115	120
acg tcc gga ggg ggg acc aag ctg gaa ata aaa c		394
Thr Ser Gly Gly Thr Lys Leu Glu Ile Lys		
125	130	

&lt;210&gt; 6

&lt;211&gt; 409

&lt;212&gt; DNA

&lt;213&gt; Mus

&lt;220&gt;

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Gly Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Asp Leu

20 25 30

gta aag cct ggg gct tca gtg aag atg tcc tgc aag gct tct gga 135

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35 40 45

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Tyr Thr Phe Val Asn His Val Met His Trp Val Lys Gln Lys Pro

50 55 60

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65 70 75

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80 85 90

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95 100 105

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Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Gly Gly Tyr

110 115 120

tat agt tac gac gac tgg ggc caa ggc acc act ctc aca gtc tcc 405

Tyr Ser Tyr Asp Asp Trp Gly Gln Gly Thr Thr Leu Thr Val Ser

125

130

135

tca g

409

Ser

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&lt;212&gt; DNA

&lt;213&gt; Mus

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10

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Gly Ser Ser Ser Asp Val Val Met Thr Gln Ser Pro Leu Ser Leu

20

25

30

cct gtc agt ctt gga gat caa gcc tcc atc tct tgc aga tca agt 135

Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser

35

40

45

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Gln Ser Leu Val His Ser Asn Gly Lys Thr Tyr Leu His Trp Tyr

50

55

60

ctg cag aag cca ggc cag tct cca aaa ctc ctg atc tac aaa gtt 225

Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val

65

70

75

tcc aac cga ttt tct ggg gtc cca gac agg ttc agt ggc agt gga 270

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提出日 平成12年 4月17日  
特願2000-115246 頁： 45/ 68

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 95 100 105  
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 Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr  
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提出日 平成12年 4月17日  
特願2000-115246 頁： 46/ 68

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65	70	75
ggt act aag tat aat gag aag ttc aag gac aag gcc act ctg act		270
Gly Thr Lys Tyr Asn Glu Lys Phe Lys Asp Lys Ala Thr Leu Thr		
80	85	90
tca gac aaa tcc tcc acc aca gcc tac atg gac ctc agc agc ctg		315
Ser Asp Lys Ser Ser Thr Thr Ala Tyr Met Asp Leu Ser Ser Leu		
95	100	105
gcc tct gag gac tct gcg gtc tat tac tgt gca aga ggg ggt tac		360
Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Gly Gly Tyr		
110	115	120
tat act tac gac gac tgg ggc caa ggc acc act ctc aca gtc tcc		405
Tyr Thr Tyr Asp Asp Trp Gly Gln Gly Thr Thr Leu Thr Val Ser		
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Ser		

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整理番号=D O J - 5 4 0 4

提出日 平成12年 4月17日  
特願2000-115246 頁： 47/ 68

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<213> Artificial Sequence

<220>

<223> PCR primer

<400> 10

cccaagcttc caccatggaa tggagctgga ta 32

<210> 11

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 11

cgcgatcca ctcacgtttt atttccagct tggt 34

<210> 12

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 12

cgcgatcca ctcacctgag gagactgtga gagt 34

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<210> 13

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 13

catgccatgg cgcgaggcca gctgcagcag 30

<210> 14

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 14

accaccacct <sup>cgtct</sup> gaggagactg tgagagt 27

<210> 15

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 15

gtctcctcag gtgggtgg ttcgggt 27

<210> 16

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 16

cacaacatcc gat<sup>cc</sup>gccac cacccga 27

<210> 17

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 17

ggcggatcgg atgttgtat gaccaa 27  
^-----taacc

<210> 18

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 18

ccggaattct cattatttat cgtcatcgtc ttttgtgtct tttatttcca gcttggt 57

<210> 19

<211> 45

<212> DNA

<213> Artificial Sequence

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&lt;220&gt;

&lt;223&gt; Linker amino acid sequence and nucleotide sequence

&lt;400&gt; 19

ggt ggt ggt ggt tcg ggt ggt ggt tcg ggt ggt ggc gga tcg	45	
Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser		
5	10	15

&lt;210&gt; 20

&lt;211&gt; 828

&lt;212&gt; DNA

&lt;213&gt; Mus

&lt;220&gt;

&lt;221&gt;CDS

&lt;222&gt;(1)...(826)

&lt;223&gt; pscM1. MABL1-scFv

&lt;400&gt; 20

atg aaa tac cta ttg cct acg gca gcc gct gga ttg tta tta ctc	45	
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu		
5	10	15

gct gcc caa cca gcc atg gcg cag gtc cag cag cag tct gga	90	
Ala Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln Ser Gly		
20	25	30

cct gac ctg gta aag cct ggg gct tca gtg aag atg tcc tgc aag	135	
Pro Asp Leu Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys		
35	40	45

gct tct gga tac acc ttc gtt aac cat gtt atg cac tgg gtg aag	180	
Ala Ser Gly Tyr Thr Phe Val Asn His Val Met His Trp Val Lys		
50	55	60

cag aag cca ggg cag ggc ctt gag tgg att gga tat att tat cct	225
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Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro  
 65 70 75  
 tac aat gat ggt act aag tac aat gag aag ttc aag ggc aag gcc 270  
 Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe Lys Gly Lys Ala  
 80 85 90  
 aca ctg act tca gag aaa tcc tcc agc gca gcc tac atg gag ctc 315  
 Thr Leu Thr Ser Glu Lys Ser Ser Ala Ala Tyr Met Glu Leu  
 95 100 105  
 agc agc ctg gcc tct gag gac tct gcg gtc tac tac tgt gca aga 360  
 Ser Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg  
 110 115 120  
 ggg ggt tac tat agt tac gac gac tgg ggc caa ggc acc act ctc 405  
 Gly Gly Tyr Tyr Ser Tyr Asp Asp Trp Gly Gln Gly Thr Thr Leu  
 125 130 135  
 aca gtc tcc tca ggt ggt ggt tcg ggt ggt ggt tcg ggt 450  
 Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly  
 140 145 150  
 ggt ggc gga tcg gat gtt gtg atg acc caa act cca ctc tcc ctg 495  
 Gly Gly Gly Ser Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu  
 155 160 165  
 cct gtc agt ctt gga gat caa gcc tcc atc tct tgc aga tct agt 540  
 Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser  
 170 175 180  
 cag agc ctt cta cac agt aaa gga aac acc tat tta caa tgg tac 585  
 Gln Ser Leu Leu His Ser Lys Gly Asn Thr Tyr Leu Gln Trp Tyr  
 185 190 195  
 cta cag aag cca ggc cag tct cca aag ctc ctg atc tac aaa gtt 630  
 Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val  
 200 205 210

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tcc aac cga ttt tct ggg gtc cca gac agg ttc agt ggc agt gga 675

Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly

215

220

225

tca ggg aca gat ttc aca ctc aag atc agc aga gtg gag gct gag 720

Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu

230

235

240

gat ctg gga gtt tat ttc tgc tct caa agt aca cat gtt ccg tac 765

Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr

245

250

255

acg tcc gga ggg ggg acc aag ctg gaa ata aaa gac tac aaa gac 810

Thr Ser Gly Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp

260

265

270

gat gac gat aaa taa tga 828

Asp Asp Asp Lys

&lt;210&gt; 21

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 21

acgcgtcgac tcccagggtcc agctgcagca g 31

&lt;210&gt; 22

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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<223> PCR primer

<400> 22

gaagggtgtat ccagaagc 18

<210> 23

<211> 819

<212> DNA

<213> Mus

<220>

<221> CDS

<222>(1)...(813)

<223> pCHOM1. MABL1-scFv

<400> 23

atg gga tgg agc tgt atc atc ctc ttc ttg gta gca aca gct aca 45

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr

5 10 15

ggt gtc gac tcc cag gtc cag ctg cag cag tct gga cct gac ctg 90

Gly Val Asp Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Asp Leu

20 25 30

gta aag cct ggg gct tca gtg aag atg tcc tgc aag gct tct gga 135

Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly

35 40 45

tac acc ttc gtt aac cat gtt atg cac tgg gtg aag cag aag cca 180

Tyr Thr Phe Val Asn His Val Met His Trp Val Lys Gln Lys Pro

50 55 60

ggg cag ggc ctt gag tgg att gga tat att tat cct tac aat gat 225

Gly Gln Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Asp

65 70 75

ggt act aag tac aat gag aag ttc aag ggc aag gcc aca ctg act 270

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Gly	Thr	Lys	Tyr	Asn	Glu	Lys	Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr
80							85					90		
tca	gag	aaa	tcc	tcc	agc	gca	gcc	tac	atg	gag	ctc	agc	agc	ctg
Ser	Glu	Lys	Ser	Ser	Ser	Ala	Ala	Tyr	Met	Glu	Leu	Ser	Ser	Leu
95								100				105		
gcc	tct	gag	gac	tct	gcg	gtc	tac	tac	tgt	gca	aga	ggg	ggt	tac
Ala	Ser	Glu	Asp	Ser	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Gly	Gly	Tyr
110								115				120		
tat	agt	tac	gac	gac	tgg	ggc	caa	ggc	acc	act	ctc	aca	gtc	tcc
Tyr	Ser	Tyr	Asp	Asp	Trp	Gly	Gln	Gly	Thr	Thr	Leu	Thr	Val	Ser
125								130				135		
tca	ggt	ggt	ggt	ggt	tcg	ggt	ggt	ggt	tcg	ggt	ggt	ggc	gga	
Ser	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	
140								145				150		
tcg	gat	gtt	gtg	atg	acc	caa	act	cca	ctc	tcc	ctg	cct	gtc	agt
Ser	Asp	Val	Val	Met	Thr	Gln	Thr	Pro	Leu	Ser	Leu	Pro	Val	Ser
155								160				165		
ctt	gga	gat	caa	gcc	tcc	atc	tct	tgc	aga	tct	agt	cag	agc	ctt
Leu	Gly	Asp	Gln	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	Leu
170								175				180		
cta	cac	agt	aaa	gga	aac	acc	tat	tta	caa	tgg	tac	cta	cag	aag
Leu	His	Ser	Lys	Gly	Asn	Thr	Tyr	Leu	Gln	Trp	Tyr	Leu	Gln	Lys
185								190				195		
cca	ggc	cag	tct	cca	aag	ctc	ctg	atc	tac	aaa	gtt	tcc	aac	cga
Pro	Gly	Gln	Ser	Pro	Lys	Leu	Leu	Ile	Tyr	Lys	Val	Ser	Asn	Arg
200								205				210		
TTT	TCT	GGG	GTC	CCA	GAC	AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA
Phe	Ser	Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr
215								220				225		

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gat ttc aca ctc aag atc agc aga gtg gag gct gag gat ctg gga 720  
 Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly  
                  230                 235                 240  
 gtt tat ttc tgc tct caa agt aca cat gtt ccg tac acg tcc gga 765  
 Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr Thr Ser Gly  
                  245                 250                 255  
 ggg ggg acc aag ctg gaa ata aaa gac tac aaa gac gat gac gat 810  
 Gly Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp Asp Asp  
                  260                 265                 270  
 aaa taa tga   819  
 Lys

&lt;210&gt; 24

&lt;211&gt; 828

&lt;212&gt; DNA

&lt;213&gt; Mus

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(822)

&lt;223&gt; pscM2. MABL2-scFv

&lt;400&gt; 24

atg aaa tac cta ttg cct acg gca gcc gct gga ttg tta tta ctc 45  
 Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu  
                  5                 10                 15

gct gcc caa cca gcc atg gcg cag gtc cag ctg cag cag tct gga 90  
 Ala Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln Ser Gly  
                  20                 25                 30

cct gaa ctg gta aag cct ggg gct tca gtg aag atg tcc tgc aag 135  
 Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys

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35	40	45
gct tct gga tac acc ttc gct aac cat gtt att cac tgg gtg aag		
Ala Ser Gly Tyr Thr Phe Ala Asn His Val Ile His Trp Val Lys		
50	55	60
cag aag cca ggg cag ggc ctt gag tgg att gga tat att tat cct		
Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro		
65	70	75
tac aat gat ggt act aag tat aat gag aag ttc aag gac aag gcc		
Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe Lys Asp Lys Ala		
80	85	90
act ctg act tca gac aaa tcc tcc acc aca gcc tac atg gac ctc		
Thr Leu Thr Ser Asp Lys Ser Ser Thr Thr Ala Tyr Met Asp Leu		
95	100	105
agc agc ctg gcc tct gag gac tct gcg gtc tat tac tgt gca aga		
Ser Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg		
110	115	120
ggg ggt tac tat act tac gac gac tgg ggc caa ggc acc act ctc		
Gly Gly Tyr Tyr Thr Tyr Asp Asp Trp Gly Gln Gly Thr Thr Leu		
125	130	135
aca gtc tcc tca ggt ggt ggt tcg ggt ggt ggt tcg ggt		
Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly		
140	145	150
ggt ggc gga tcg gat gtt gtg atg acc caa agt cca ctc tcc ctg		
Gly Gly Gly Ser Asp Val Val Met Thr Gln Ser Pro Leu Ser Leu		
155	160	165
cct gtc agt ctt gga gat caa gcc tcc atc tct tgc aga tca agt		
Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser		
170	175	180
cag agc ctt gtg cac agt aat gga aag acc tat tta cat tgg tac		

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Gln Ser Leu Val His Ser Asn Gly Lys Thr Tyr		
185	190	195
ctg cag aag cca ggc cag tct cca aaa ctc ctg atc tac aaa gtt		630
Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val		
200	205	210
tcc aac cga ttt tct ggg gtc cca gac agg ttc agt ggc agt gga		675
Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly		
215	220	225
tca gtg aca gat ttc aca ctc atg atc agc aga gtg gag gct gag		720
Ser Val Thr Asp Phe Thr Leu Met Ile Ser Arg Val Glu Ala Glu		
230	235	240
gat ctg gga gtt tat ttc tgc tct caa agt aca cat gtt ccg tac		765
Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr		
245	250	255
acg ttc gga ggg ggg acc aag ctg gaa ata aaa gac tac aaa gac		810
Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp		
260	265	270
gat gac gat aaa taa tga		828
Asp Asp Asp Lys		

&lt;210&gt; 25

&lt;211&gt; 819

&lt;212&gt; DNA

&lt;213&gt; Mus

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(813)

&lt;223&gt; pCHOM2. MABL2-scFv

&lt;400&gt; 25

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atg gga tgg agc tgt atc atc ctc ttc ttg gta gca aca gct aca	45
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr	
5 10 15	
ggt gtc gac tcc cag gtc cag ctg cag cag tct gga cct gaa ctg	90
Gly Val Asp Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu	
20 25 30	
gta aag cct ggg gct tca gtg aag atg tcc tgc aag gct tct gga	135
Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly	
35 40 45	
tac acc ttc gct aac cat gtt att cac tgg gtg aag cag aag cca	180
Tyr Thr Phe Ala Asn His Val Ile His Trp Val Lys Gln Lys Pro	
50 55 60	
ggg cag ggc ctt gag tgg att gga tat att tat cct tac aat gat	225
Gly Gln Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Asp	
65 70 75	
ggt act aag tat aat gag aag ttc aag gac aag gcc act ctg act	270
Gly Thr Lys Tyr Asn Glu Lys Phe Lys Asp Lys Ala Thr Leu Thr	
80 85 90	
tca gac aaa tcc tcc acc aca gcc tac atg gac ctc agc agc ctg	315
Ser Asp Lys Ser Ser Thr Thr Ala Tyr Met Asp Leu Ser Ser Leu	
95 100 105	
gcc tct gag gac tct gcg gtc tat tac tgt gca aga ggg ggt tac	360
Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Gly Gly Tyr	
110 115 120	
tat act tac gac gac tgg ggc caa ggc acc act ctc aca gtc tcc	405
Tyr Thr Tyr Asp Asp Trp Gly Gln Gly Thr Thr Leu Thr Val Ser	
125 130 135	
tca ggt ggt ggt tcg ggt ggt ggt tcg ggt ggt ggc gga	450
Ser Gly Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Gly Gly	

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140	145	150
tcg gat gtt gtg atg acc caa agt cca ctc tcc ctg cct gtc agt		495
Ser Asp Val Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Ser		
155	160	165
ctt gga gat caa gcc tcc atc tct tgc aga tca agt cag agc ctt		540
Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu		
170	175	180
gtg cac agt aat gga aag acc tat tta cat tgg tac ctg cag aag		585
Val His Ser Asn Gly Lys Thr Tyr Leu His Trp Tyr Leu Gln Lys		
185	190	195
cca ggc cag tct cca aaa ctc ctg atc tac aaa gtt tcc aac cga		630
Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg		
200	205	210
ttt tct ggg gtc cca gac agg ttc agt ggc agt gga tca gtg aca		675
Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Val Thr		
215	220	225
gat ttc aca ctc atg atc agc aga gtg gag gct gag gat ctg gga		720
Asp Phe Thr Leu Met Ile Ser Arg Val Glu Ala Glu Asp Leu Gly		
230	235	240
gtt tat ttc tgc tct caa agt aca cat gtt ccg tac acg ttc gga		765
Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr Thr Phe Gly		
245	250	255
ggg ggg acc aag ctg gaa ata aaa gac tac aaa gac gat gac gat		810
Gly Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp Asp Asp		
260	265	270
aaa taa tga		819
Lys		

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&lt;211&gt; 456

&lt;212&gt; DNA

&lt;213&gt; Mus

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(450)

&lt;223&gt; pCHO-shIAP. Soluble human IAP

&lt;400&gt; 26

atg tgg ccc ctg gta gcg gcg ctg ttg ctg ggc tcg gcg tgc tgc 45

Met Trp Pro Leu Val Ala Ala Leu Leu Leu Gly Ser Ala Cys Cys

5 10 15

gga tca gct cag cta cta ttt aat aaa aca aaa tct gta gaa ttc 90

Gly Ser Ala Gln Leu Leu Phe Asn Lys Thr Lys Ser Val Glu Phe

20 25 30

acg ttt tgt aat gac act gtc gtc att cca tgc ttt gtt act aat 135

Thr Phe Cys Asn Asp Thr Val Val Ile Pro Cys Phe Val Thr Asn

35 40 45

atg gag gca caa aac act act gaa gta tac gta aag tgg aaa ttt 180

Met Glu Ala Gln Asn Thr Thr Glu Val Tyr Val Lys Trp Lys Phe

50 55 60

aaa gga aga gat att tac acc ttt gat gga gct cta aac aag tcc 225

Lys Gly Arg Asp Ile Tyr Thr Phe Asp Gly Ala Leu Asn Lys Ser

65 70 75

act gtc ccc act gac ttt agt agt gca aaa att gaa gtc tca caa 270

Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser Gln

80 85 90

tta cta aaa gga gat gcc tct ttg aag atg gat aag agt gat gct 315

Leu Leu Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser Asp Ala

95 100 105

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gtc tca cac aca gga aac tac act tgt gaa gta aca gaa tta acc 360

Val Ser His Thr Gly Asn Tyr Thr Cys Glu Val Thr Glu Leu Thr

110 115 120

aga gaa ggt gaa acg atc atc gag cta aaa tat cgt gtt gtt tca 405

Arg Glu Gly Glu Thr Ile Ile Glu Leu Lys Tyr Arg Val Val Ser

125 130 135

tgg ttt tct cca aat gaa aat gac tac aag gac gac gat gac aag 450

Trp Phe Ser Pro Asn Glu Asn Asp Tyr Lys Asp Asp Asp Lys

140 145 150

tga tag 456

&lt;210&gt; 27

&lt;211&gt; 46

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 27

ggaattccat atgcaagtgc aacttcaaca gtctggacct gaactg 46

&lt;210&gt; 28

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 28

ggaattctca ttattttatt tccagcttgg t 31

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<210> 29

<211> 741

<212> DNA

<213> Mus

<220>

<221> CDS

<222> (1)...(735)

<223> pscM2DEM02. MABL2-scFv

<400> 29

atg caa gtg caa ctt caa cag tct gga cct gaa ctg gta aag cct 45

Met Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro

5 10 15

ggg gct tca gtg aag atg tcc tgc aag gct tct gga tac acc ttc 90

Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe

20 25 30

gct aac cat gtt att cac tgg gtg aag cag aag cca ggg cag ggc 135

Ala Asn His Val Ile His Trp Val Lys Gln Lys Pro Gly Gln Gly

35 40 45

ctt gag tgg att gga tat att tat cct tac aat gat ggt act aag 180

Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Asp Gly Thr Lys

50 55 60

tat aat gag aag ttc aag gac aag gcc act ctg act tca gac aaa 225

Tyr Asn Glu Lys Phe Lys Asp Lys Ala Thr Leu Thr Ser Asp Lys

65 70 75

tcc tcc acc aca gcc tac atg gac ctc agc agc ctg gcc tct gag 270

Ser Ser Thr Thr Ala Tyr Met Asp Leu Ser Ser Leu Ala Ser Glu

80 85 90

gac tct gcg gtc tat tac tgt gca aga ggg ggt tac tat act tac 315

Asp Ser Ala Val Tyr Tyr Cys Ala Arg Gly Gly Tyr Tyr Thr Tyr

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95	100	105
gac gac tgg ggc caa ggc acc act ctc aca gtc tcc tca ggt ggt 360		
Asp Asp Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Gly Gly		
110	115	120
ggt ggt tcg ggt ggt ggt tcg ggt ggt ggc gga tcg gat gtt 405		
Gly Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Asp Val		
125	130	135
gtg atg acc caa agt cca ctc tcc ctg cct gtc agt ctt gga gat 450		
Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly Asp		
140	145	150
caa gcc tcc atc tct tgc aga tca agt cag agc ctt gtg cac agt 495		
Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser		
155	160	165
aat gga aag acc tat tta cat tgg tac ctg cag aag cca ggc cag 540		
Asn Gly Lys Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln		
170	175	180
tct cca aaa ctc ctg atc tac aaa gtt tcc aac cga ttt tct ggg 585		
Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly		
185	190	195
gtc cca gac agg ttc agt ggc agt gga tca gtg aca gat ttc aca 630		
Val Pro Asp Arg Phe Ser Gly Ser Val Thr Asp Phe Thr		
200	205	210
ctc atg atc agc aga gtg gag gct gag gat ctg gga gtt tat ttc 675		
Leu Met Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe		
215	220	225
tgc tct caa agt aca cat gtt ccg tac acg ttc gga ggg ggg acc 720		
Cys Ser Gln Ser Thr His Val Pro Tyr Thr Phe Gly Gly Thr		
230	235	240
aag ctg gaa ata aaa taa tga 741		

Lys Leu Glu Ile Lys

**【図面の簡単な説明】**

**【図1】**

ヒトIgG1抗体が、ヒトIAPを発現するL1210細胞(hIAP/L1210)に結合しないことを示すフローサイトメトリーの結果を示す図である。

**【図2】**

キメラMABL-1抗体が、ヒトIAPを発現するL1210細胞(hIAP/L1210)に特異的に結合することを示すフローサイトメトリーの結果を示す図である。

**【図3】**

キメラMABL-2抗体が、ヒトIAPを発現するL1210細胞(hIAP/L1210)に特異的に結合することを示すフローサイトメトリーの結果を示す図である。

**【図4】**

本発明にかかる一本鎖Fvの作成方法を模式的に示す図である。

**【図5】**

本発明の一本鎖FvをコードするDNAを、大腸菌にて発現させるために使用可能な発現プラスミドの一例の構造を示す。

**【図6】**

本発明の一本鎖FvをコードするDNAを、哺乳動物細胞にて発現させるために使用する発現プラスミドの一例の構造を示す。

**【図7】**

実施例5.4で得られたウエスタンプロットの結果を示す写真である。左側より、分子量マーカー(上から97.4、66、45、31、21.5、14.5kDaを示す)、pCHO1導入COS7細胞培養上清、pCHOM2導入細胞培養上清。pCHOM2導入細胞培養上清に再構成MABL-2抗体一本鎖Fv(矢印)が明らかに含まれていることを示す。

**【図8】**

**[Brief Description of the Drawings]**

Figure 1 shows the result of flow cytometry, illustrating that human IgG antibody does not bind to L1210 cells expressing human IAP (hIAP/L1210).

5           Figure 2 shows the result of flow cytometry, illustrating that the chimera MABL-1 antibody specifically binds to L1210 cells expressing human IAP (hIAP/L1210).

10          Figure 3 shows the result of flow cytometry, illustrating that the chimera MABL-2 antibody specifically binds to L1210 cells expressing human IAP (hIAP/L1210).

Figure 4 schematically illustrates the process for producing the single chain Fv according to the present invention.

15          Figure 5 illustrates a structure of an expression plasmid which can be used to express a DNA encoding the single chain Fv of the invention in E. coli.

Figure 6 illustrates a structure of an expression plasmid which is used to express a DNA encoding the single chain Fv of the invention in mammalian cells.

20          Figure 7 shows a photograph showing the result of western blotting in Example 5.4. From the left, a molecular weight marker (which indicates 97.4, 66, 45, 31, 21.5 and 14.5 kDa from the top), the culture supernatant of pCHO1-introduced COS7 cells and the culture supernatant of pCHOM2-introduced COS7 cells. It illustrates that the reconstructed single chain Fv of the antibody MABL-2 (arrow) is contained in the culture supernatant of the pCHOM2-introduced cells.

Figure 8 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of pCHO1/COS7 cell as a control does not bind to pCOS1/L1210 cell as a control.

5                 Figure 9 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of MABL2-scFv/COS7 cells does not bind to pCOS1/L1210 cells as a control.

10                Figure 10 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of pCOS1/COS7 cells as a control does not bind to hIAP/L1210 cells.

15                Figure 11 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically binds to hIAP/L1210 cells.

20                Figure 12 shows the result of the competitive ELISA in Example 5.6, wherein the binding activity of the single chain Fv of the invention (MABL2-scFv) to the antigen is demonstrated in terms of the inhibition of binding of the mouse monoclonal antibody MABL-2 to the antigen as an index, in comparison with the culture supernatant of pCHO1/COS7 cells as a control.

25                Figure 13 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a

control does not induce the apoptosis of pCOS1/L1210 cells as a control.

Figure 14 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells does not induce apoptosis of pCOS1/L1210 cells as a control.

Figure 15 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a control does not induce apoptosis of hIAP/L1210 cells.

Figure 16 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically induces apoptosis of hIAP/L1210 cells.

Figure 17 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a control does not induce apoptosis of CCRF-CEM cells (at 50% of the final concentration).

Figure 18 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically induces apoptosis of CCRF-CEM cells (at 50% of the final concentration).

Figure 19 shows the chromatogram obtained in the purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells in Example 5.9,

illustrating that fraction A and fraction B were obtained as the major peaks when the fraction from Blue-sepharose column was purified with hydroxyapatite column.

Figure 20 shows the results of purification by gel filtration of fraction A and fraction B obtained in Example 5.9-(2), illustrating that the major peaks (AI and BI, respectively) were eluted from fraction A at approximately 36 kD of the apparent molecular weight and from fraction B at approximately 76 kD.

Figure 21 is the analysis on SDS-PAGE of the fractions obtained in the purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells in Example 5.9, illustrating that a single band of approximately 35 kD of molecular weight was observed in both fractions.

Figure 22 shows the results of analysis of fractions AI and BI obtained by gel filtration in the purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells, wherein fraction AI comprises monomer and fraction BI comprises dimer.

Figure 23 illustrates a structure of an expression plasmid which can be used to express a DNA encoding the single chain Fv of the invention in E. coli.

Figure 24 shows the results of purification on the gel filtration column of crude products of the single chain Fv polypeptide derived from the antibody MABL-2 produced by E. coli obtained in Example 5.12, wherein each peak

indicates monomer or dimer, respectively, of the single chain Fv produced by E. coli.

5

Figure 25 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that mouse IgG antibody as a control does not induce apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

10

Figure 26 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that the dimer of MABL2-scFv produced by the CHO cells remarkably induces apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

15

Figure 27 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that the dimer of MABL2-scFv produced by E. coli remarkably induces apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

20

Figure 28 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that apoptosis induction to hIAP/L1210 cells by the MABL2-scFv monomer produced by the CHO cells is the same level as that of the control (the final concentration of 3  $\mu$ g/ml).

25

Figure 29 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that apoptosis induction to hIAP/L1210 cells of the MABL2-scFv monomer produced by E. coli is the same level as that of control (the final concentration of 3  $\mu$ g/ml).

Figure 30 shows the results of quantitative measurement of human IgG in the serum of a human myeloma cell line KPMM2-transplanted mouse, indicating amounts of human IgG produced by the human myeloma cells in the mouse.

5 It illustrates that the dimer of scFv/CHO remarkably inhibited growth of the KPMM2 cells.

Figure 31 shows the survival time of the mouse after the transplantation of tumor, illustrating that the scFv/CHO dimer-administered group elongated remarkably the 10 survival time.

Figure 32 schematically illustrates a polypeptide of this invention which two H chain V regions and two L chain V regions are connected through a linker.

[Name of Document]

**ABSTRACT**

[Abstract]

[Problem(s) to be Solved by the Invention]

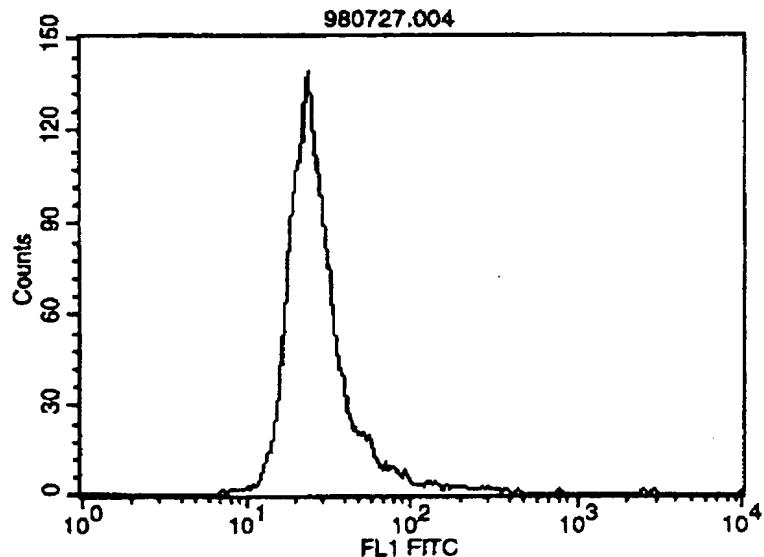
It is to provide reconstructed polypeptides  
5 characterized by inducing apoptosis in nuclear blood cells  
having human integrin associated protein (IAP) without  
causing hemagglutination.

[Means for Solving the Problem]

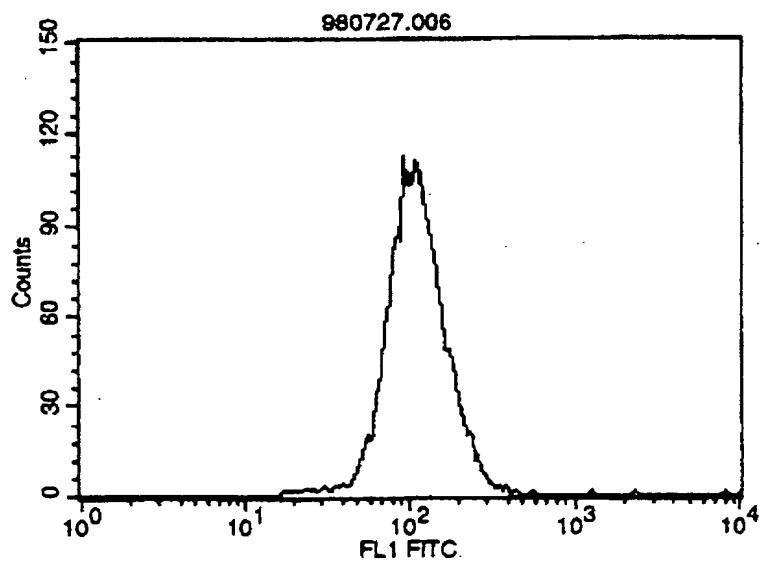
This reconstructed polypeptide contains H chain V  
10 region and L chain V region of a monoclonal antibody which  
induces apoptosis in nuclear blood cells having IAP,  
preferably human IAP. This reconstructed polypeptide is  
useful as a remedy for blood diseases such as leukemia.

【書類名】図面 [Name of Document] Drawing(s)

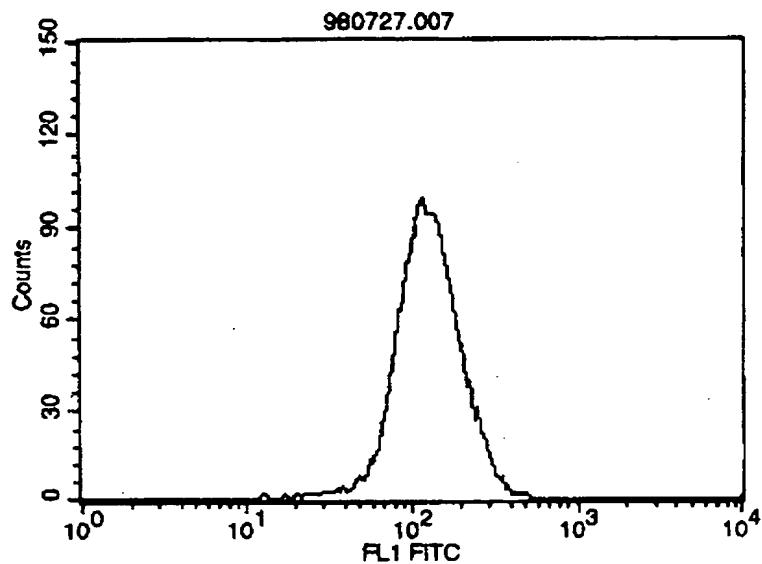
【図1】Fig.1



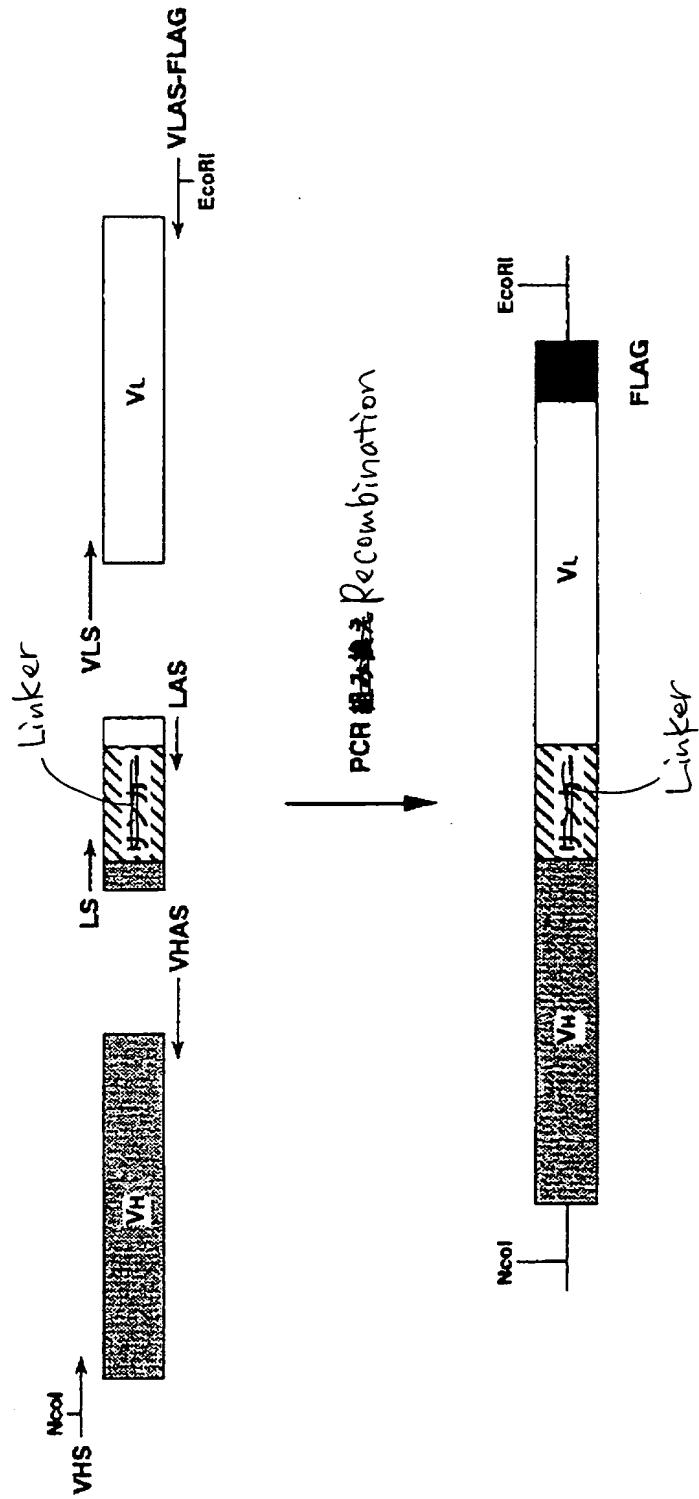
【図2】Fig.2



【図3】Fig.3



【図4】Fig. 4.



【図5】Fig.5

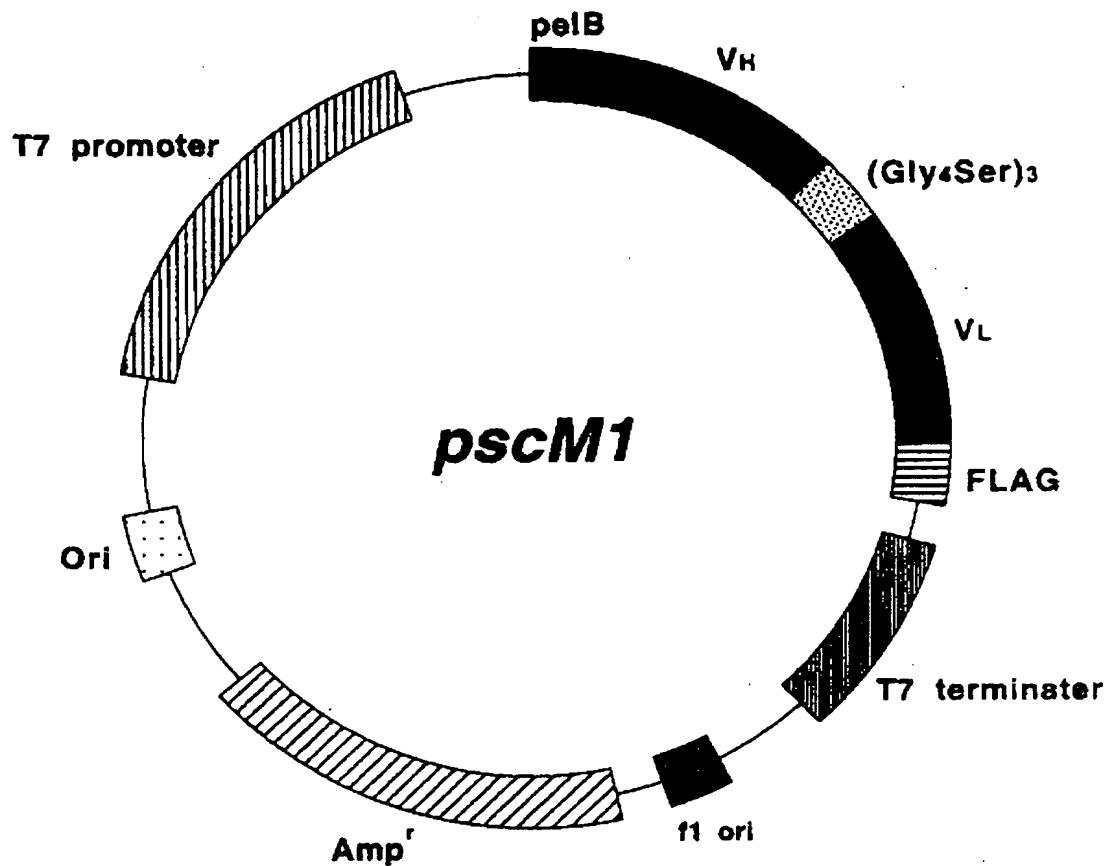
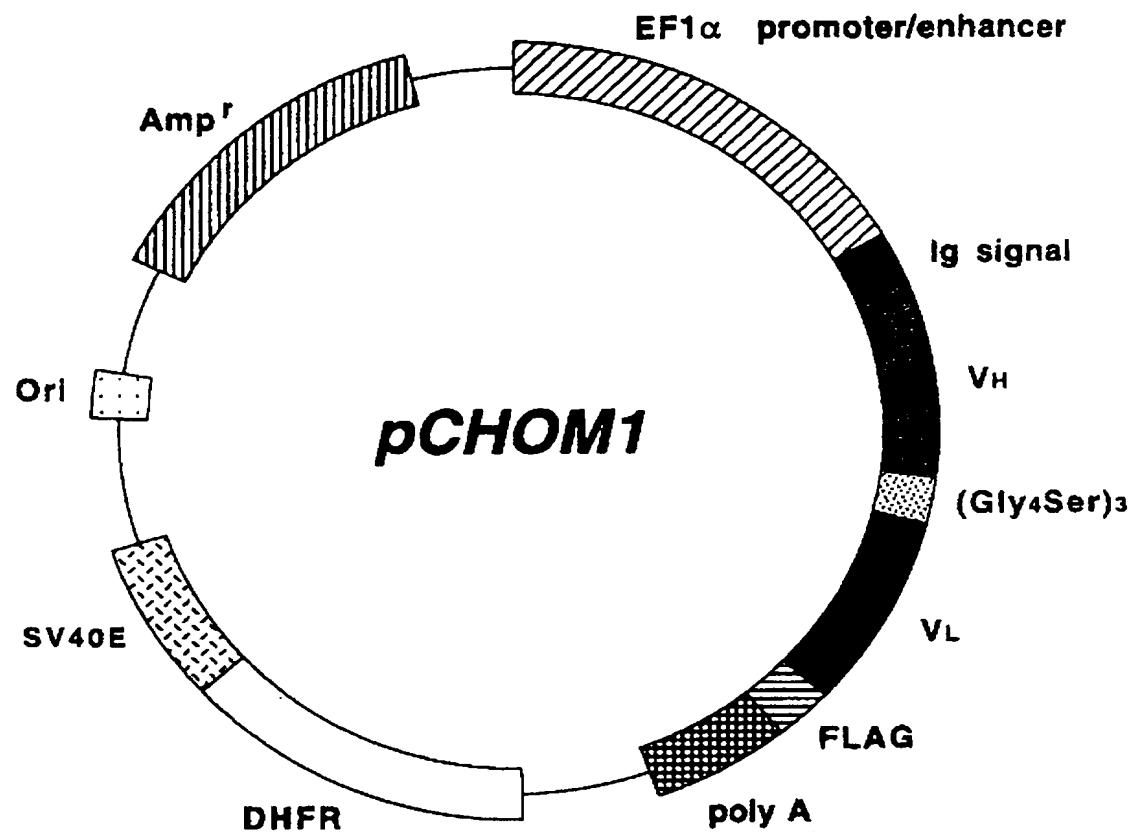
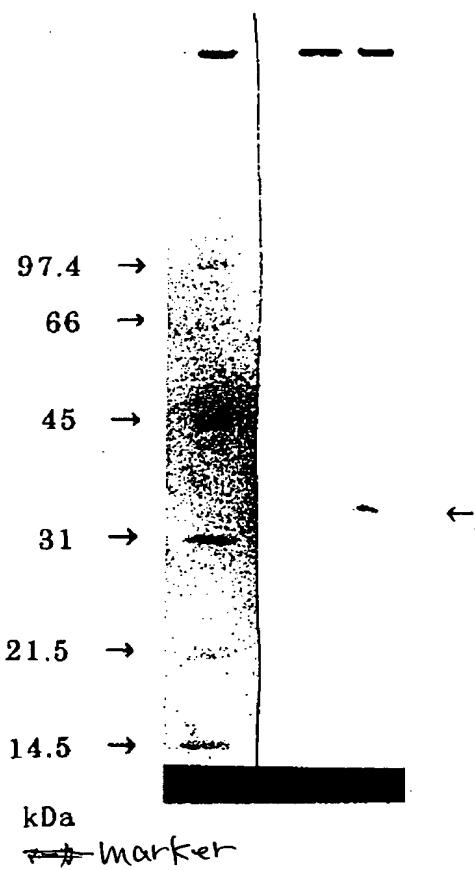


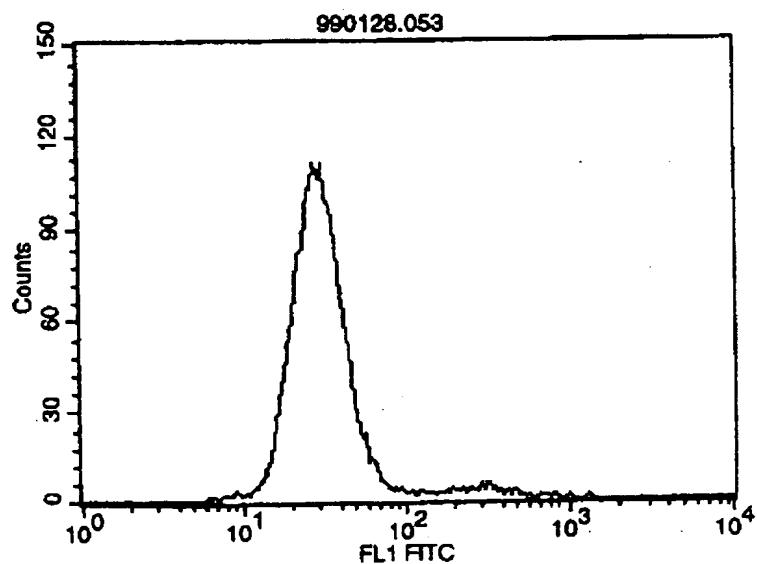
図6 Fig.6



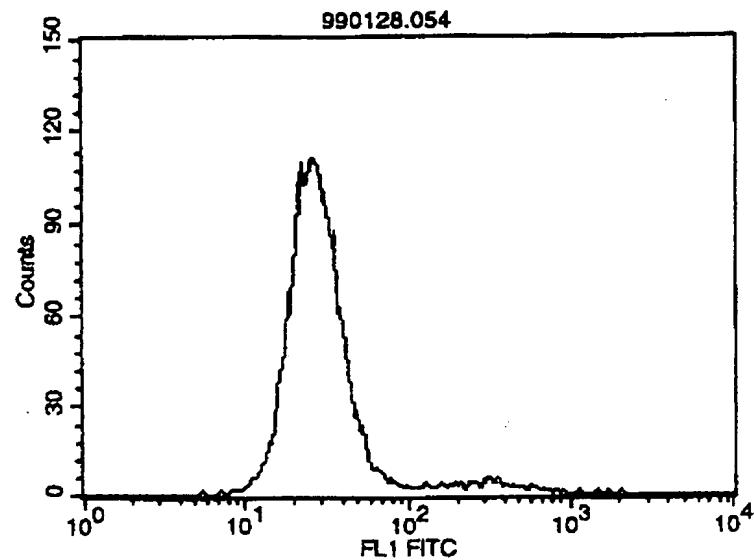
【図7】 Fig. 7



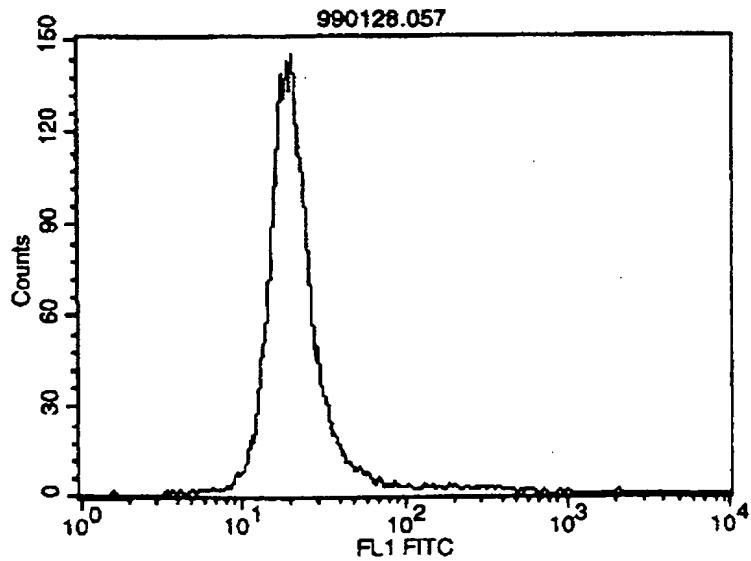
【図8】 Fig. 8



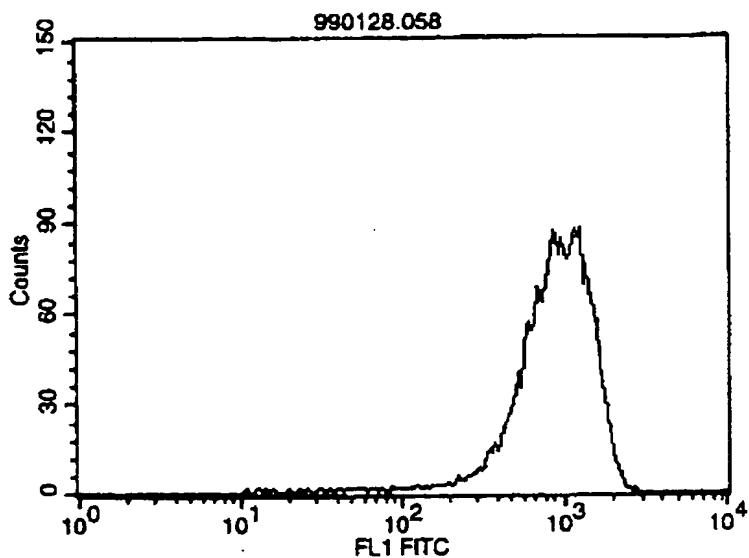
【図9】Fig.9



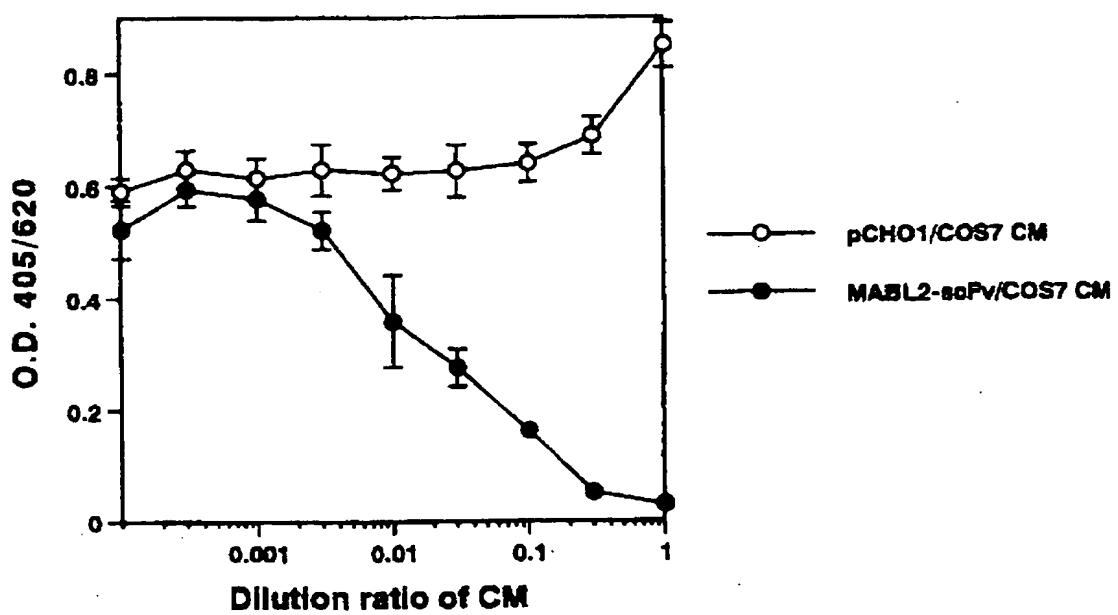
【図10】Fig.10



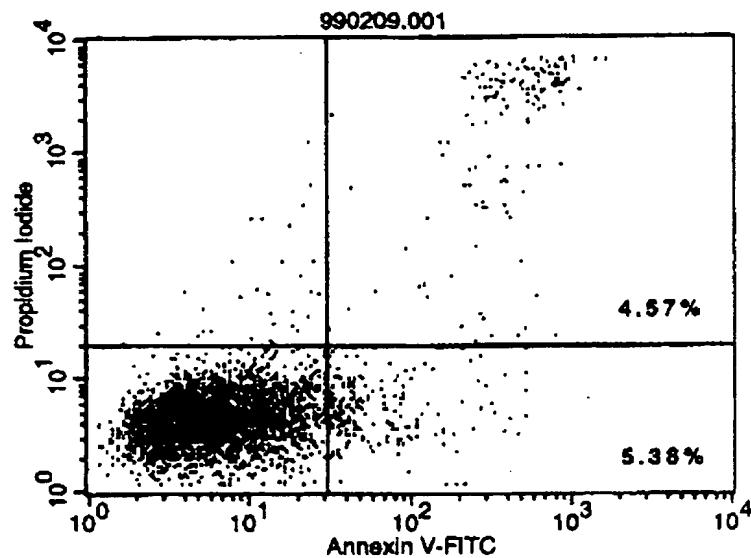
【図1-1】 Fig.11



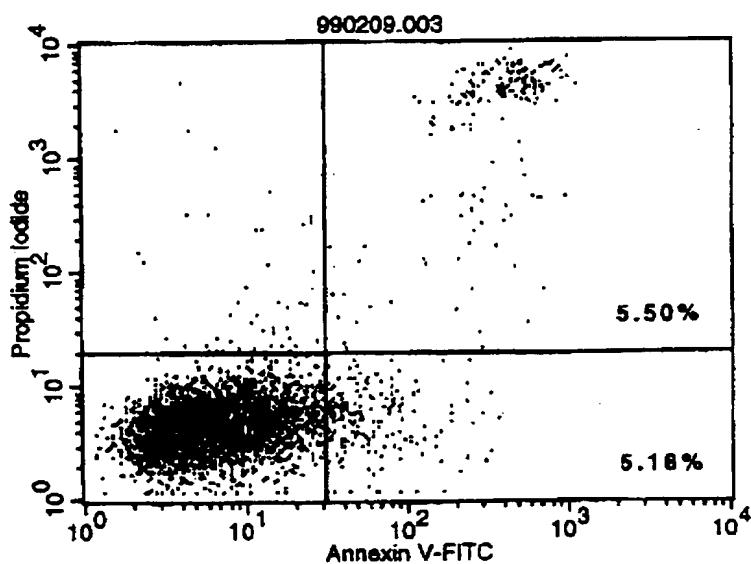
【図1-2】 Fig.12

**Competitive ELISA**

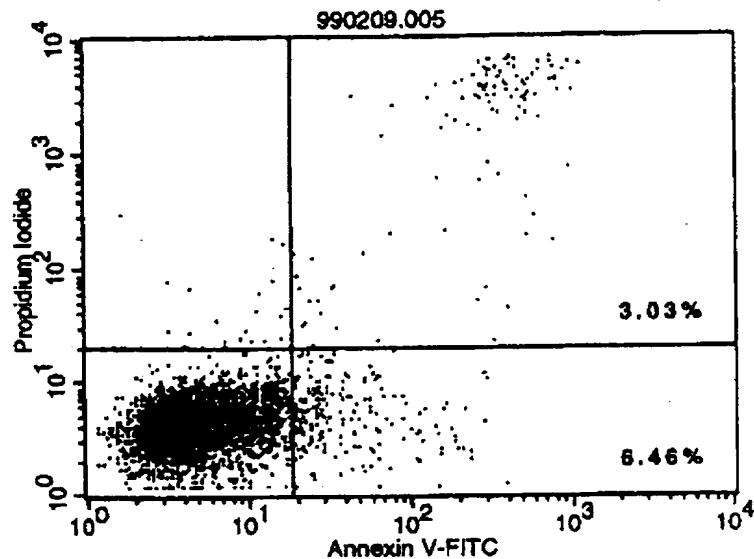
【図13】Fig. 13



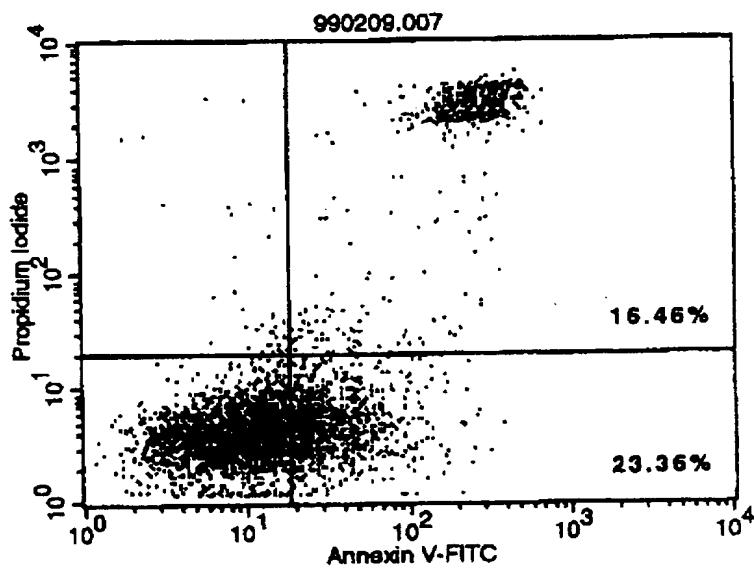
【図14】Fig. 14



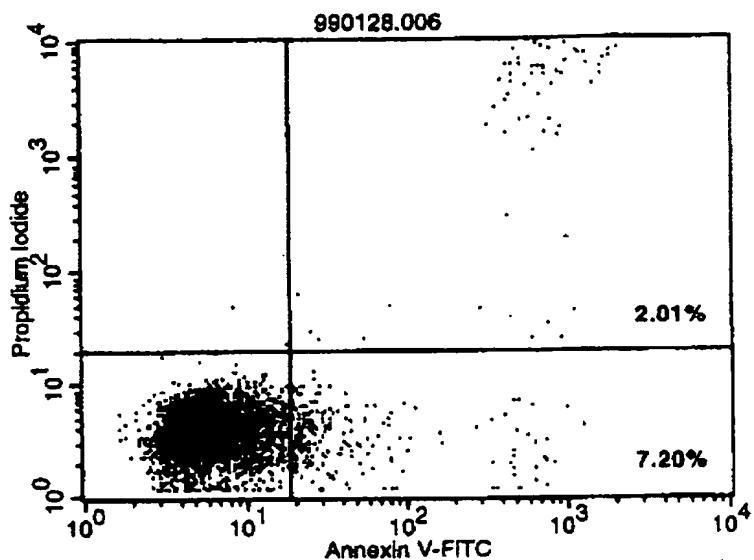
[図1-5] Fig. 15



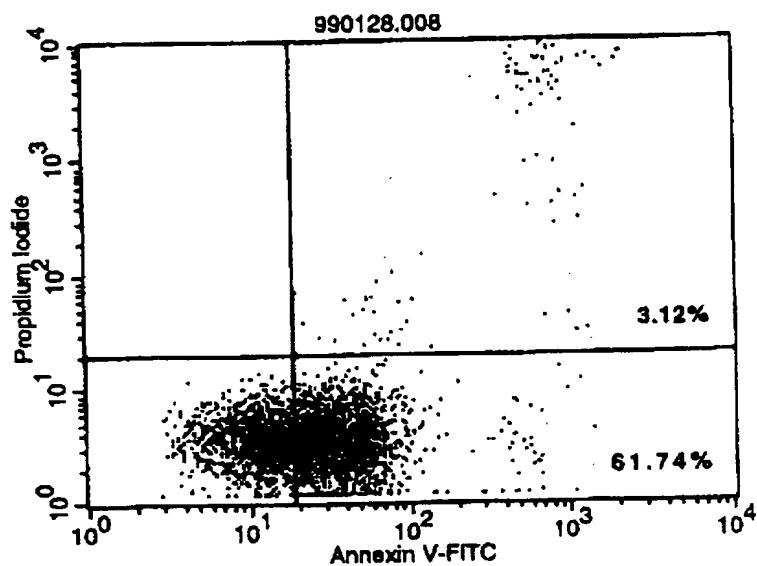
[図1-6] Fig. 16



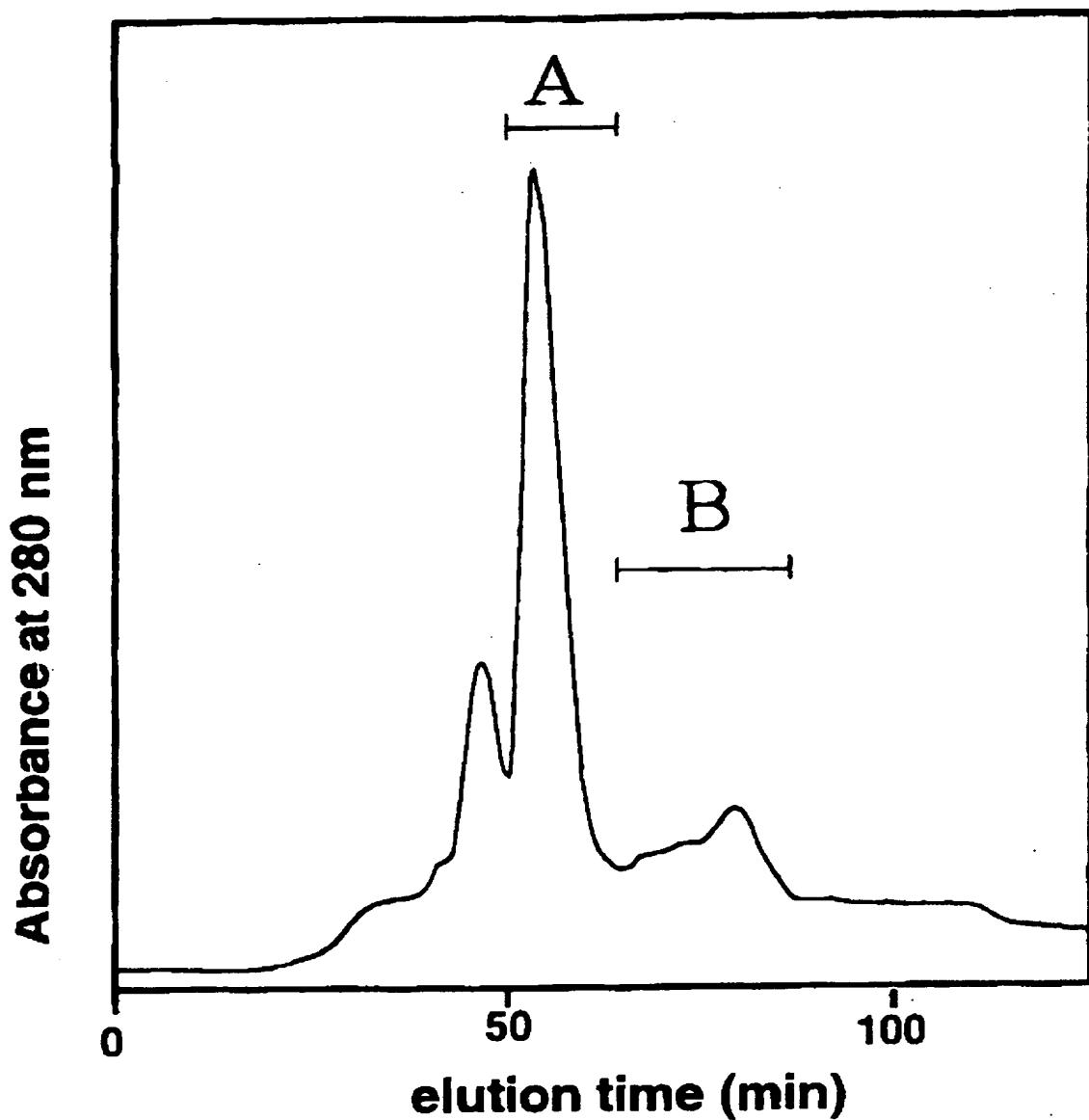
【図1-7】 Fig.17



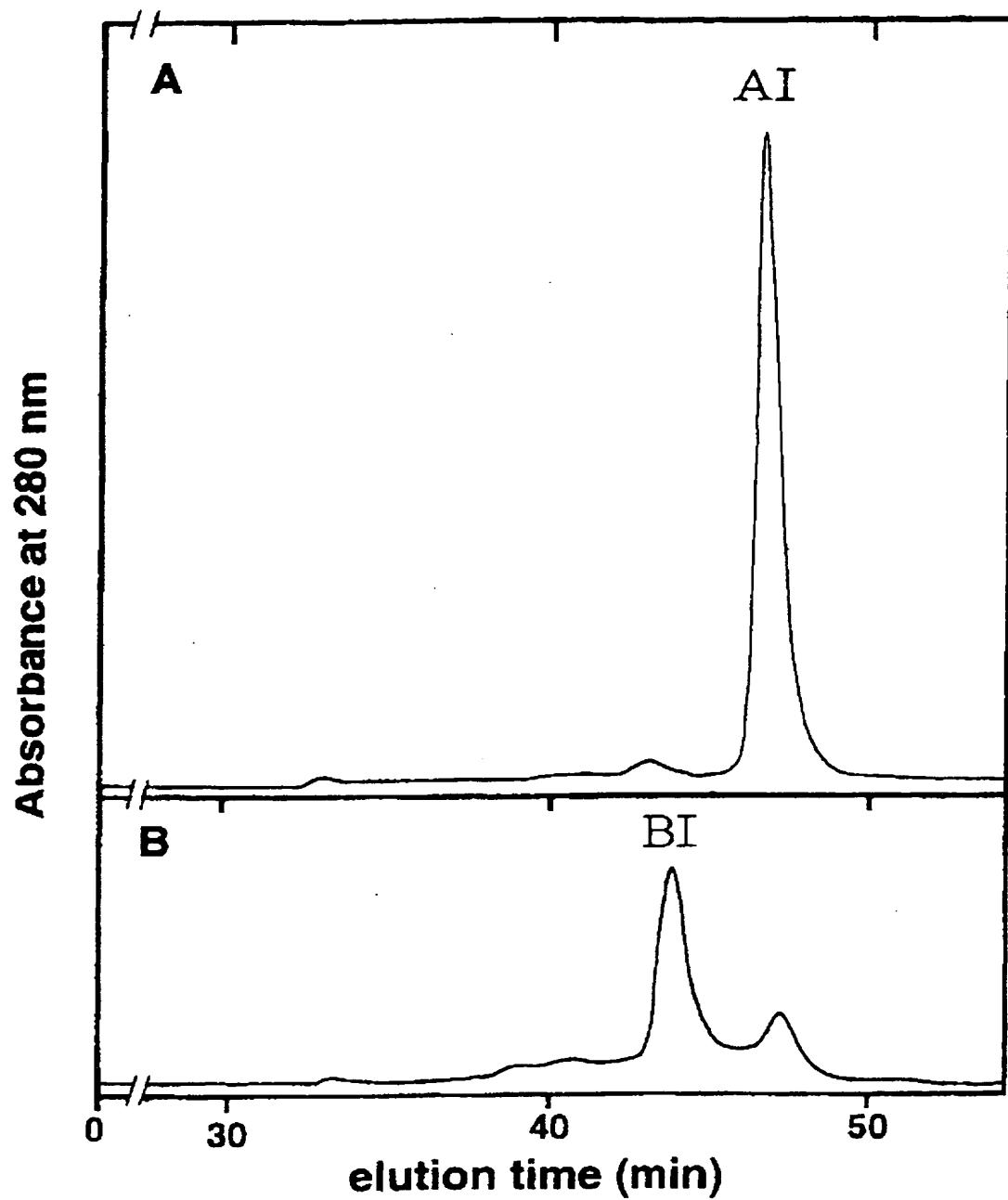
【図1-8】 Fig.18



【図19】Fig.19



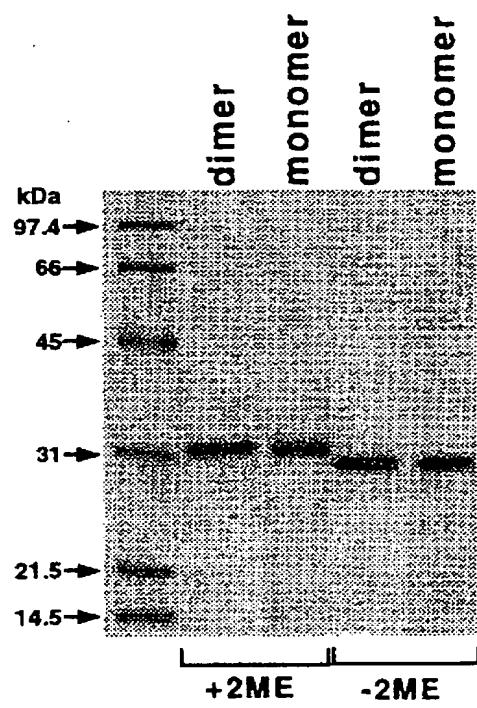
【図20】Fig.20



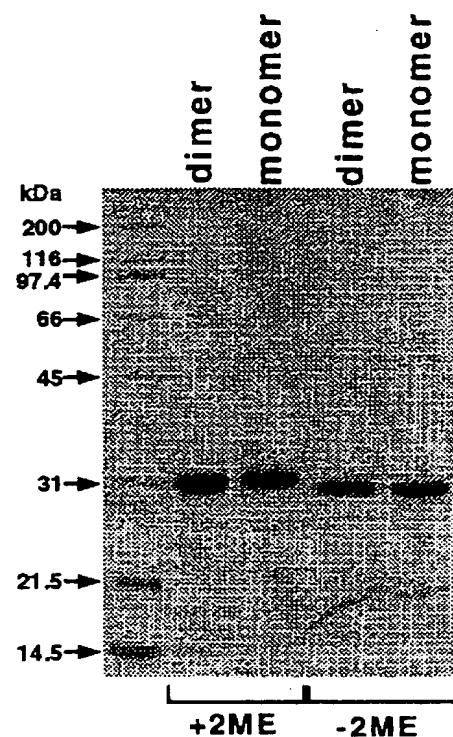
【図2-1】 Fig.21

**SDS-PAGE analysis of MABL2-scFv**

&lt;CHO&gt;

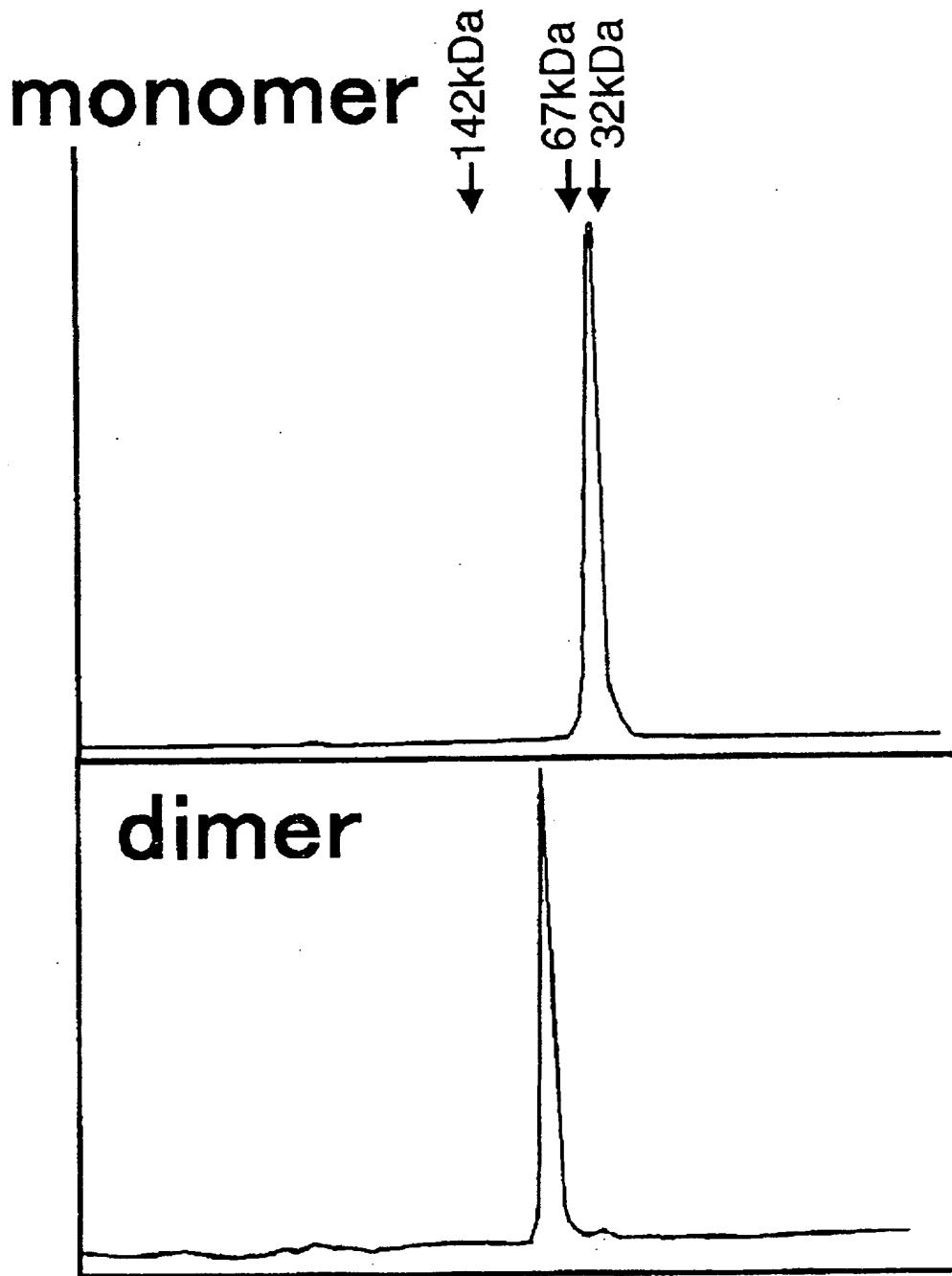


&lt;E. coli&gt;

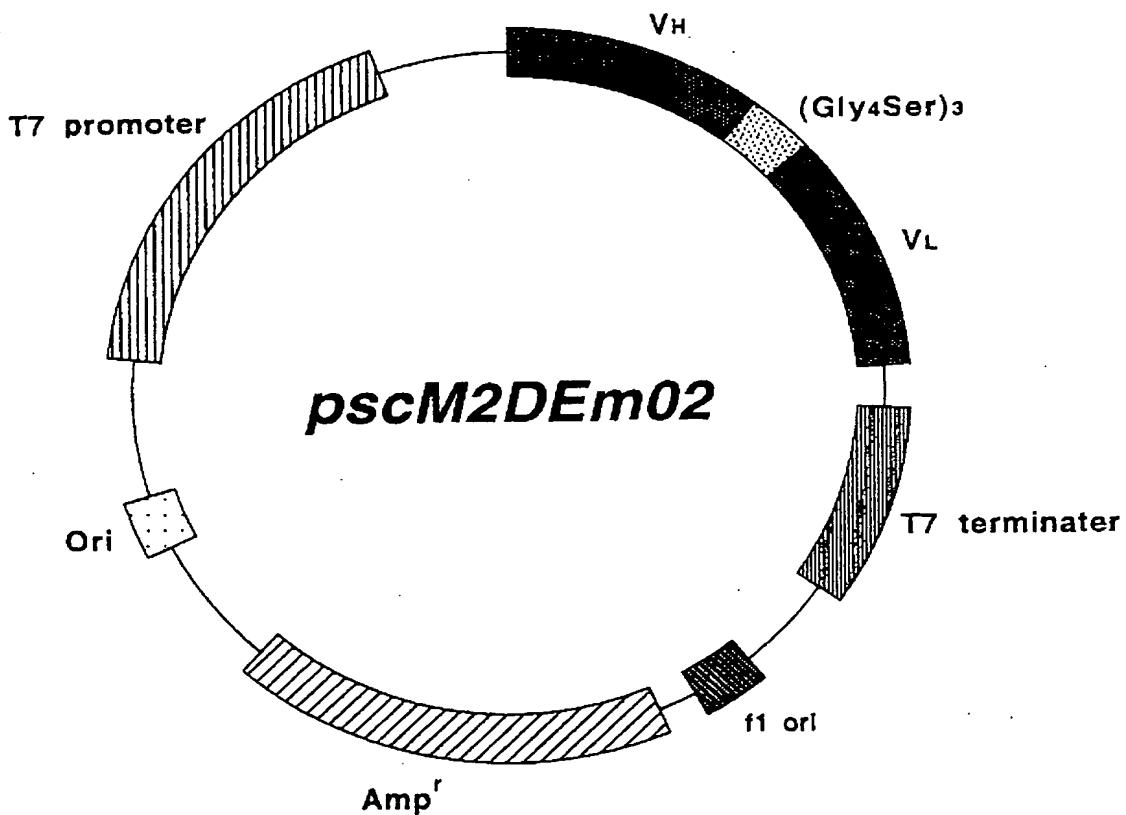


【図2-2】~~Figs. 2-2~~

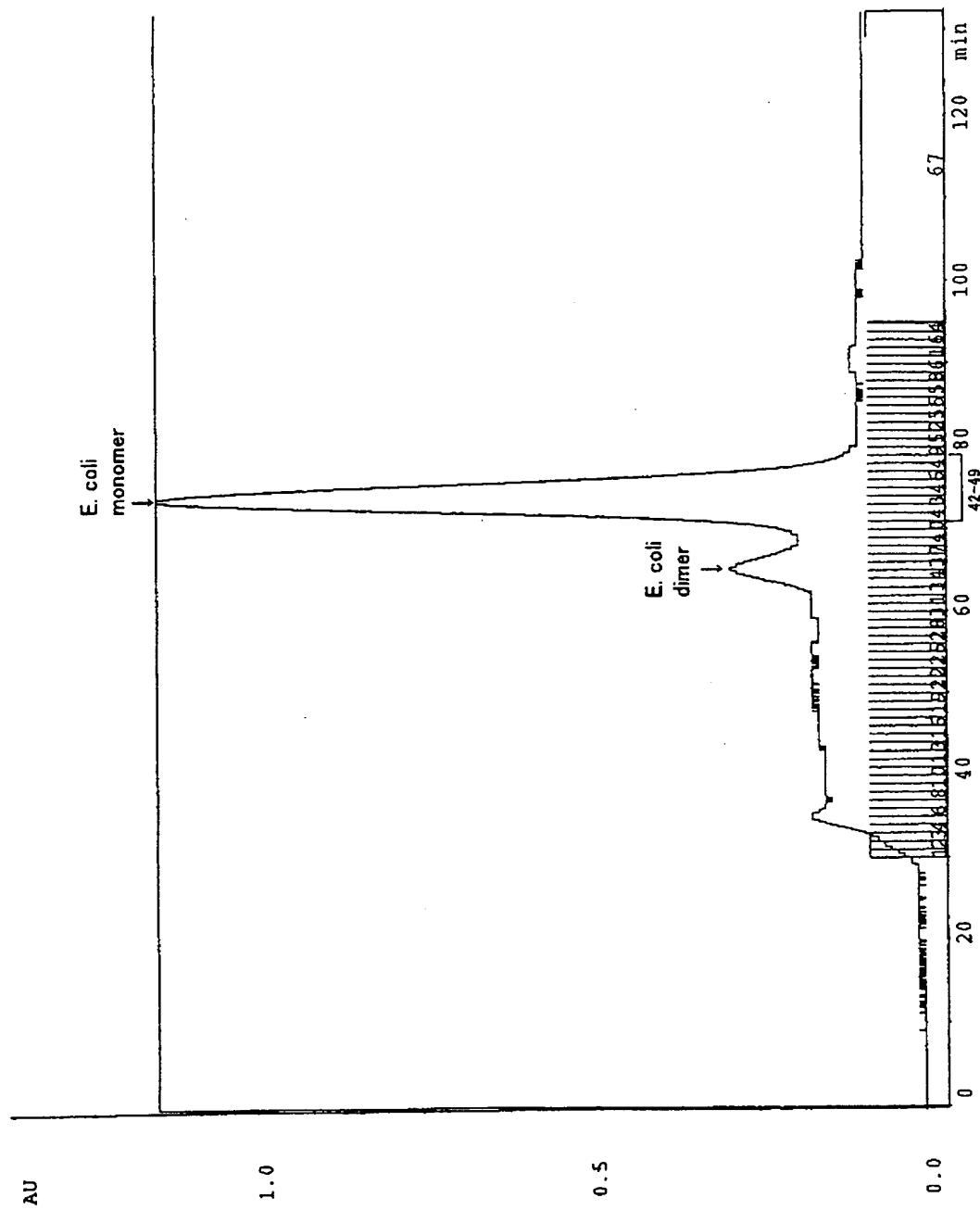
TSK gel G3000SW  
20 mM Acetate buffer, 0.15 M NaCl, pH 6.0



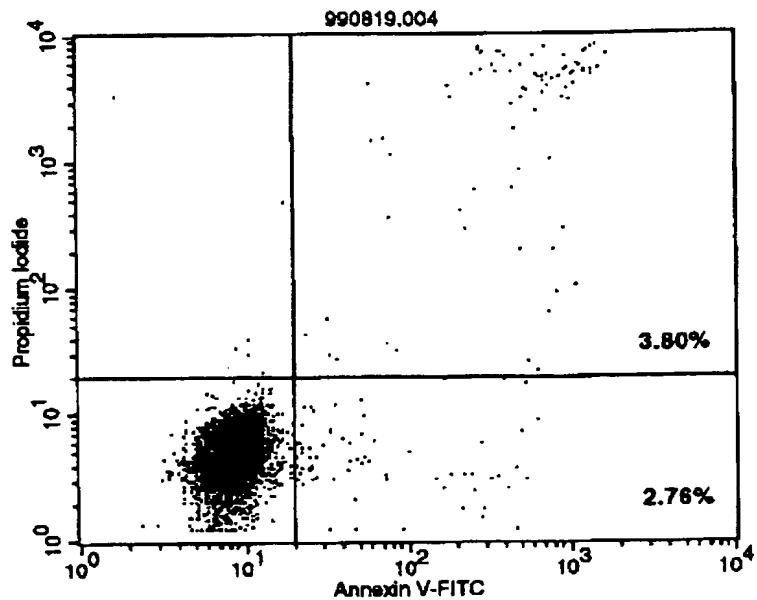
【図2-3】 Fig. 2-3



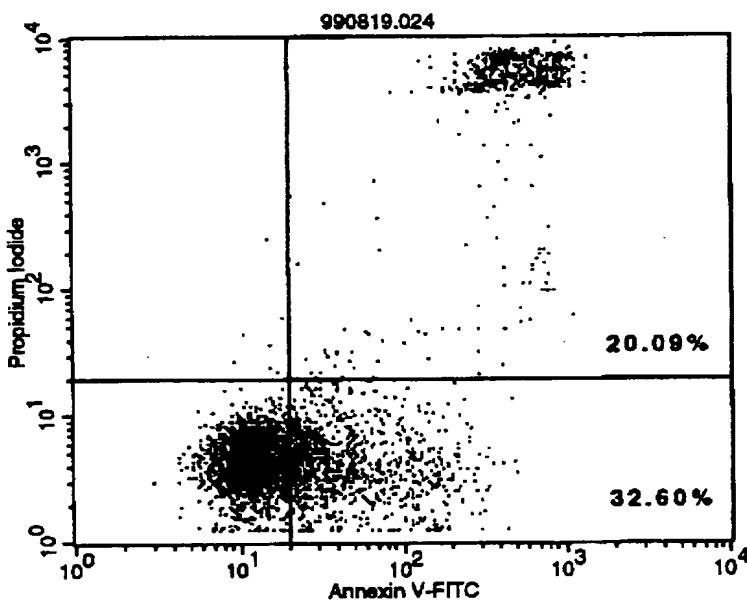
【図2-4】Fig. 24



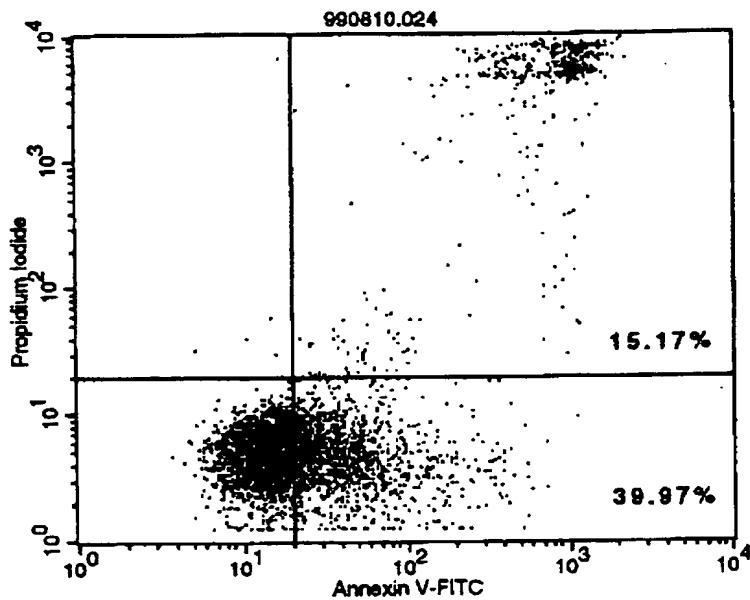
【図2-5】 Fig.25



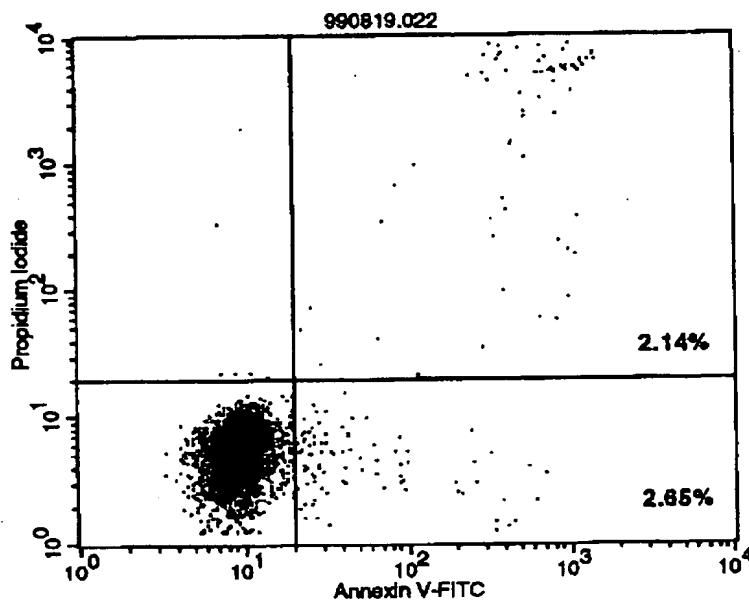
【図2-6】 Fig.26



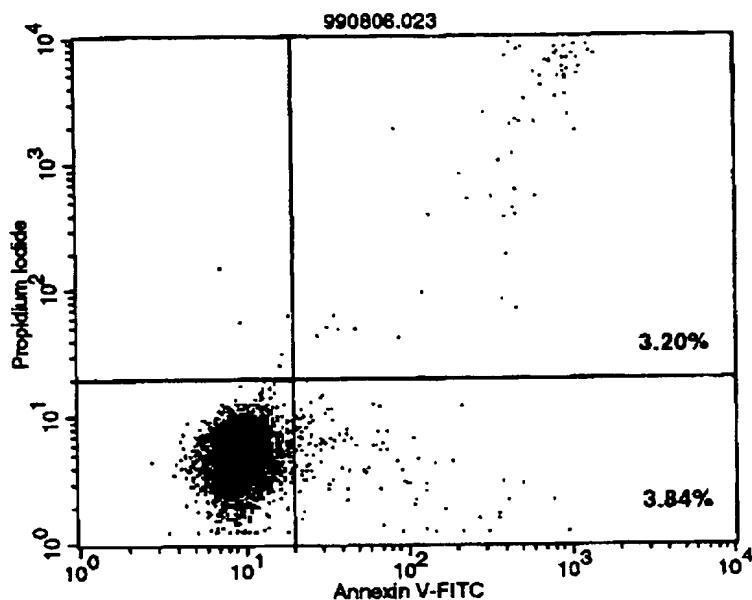
【図2-7】 Fig.27



【図2-8】 Fig.28

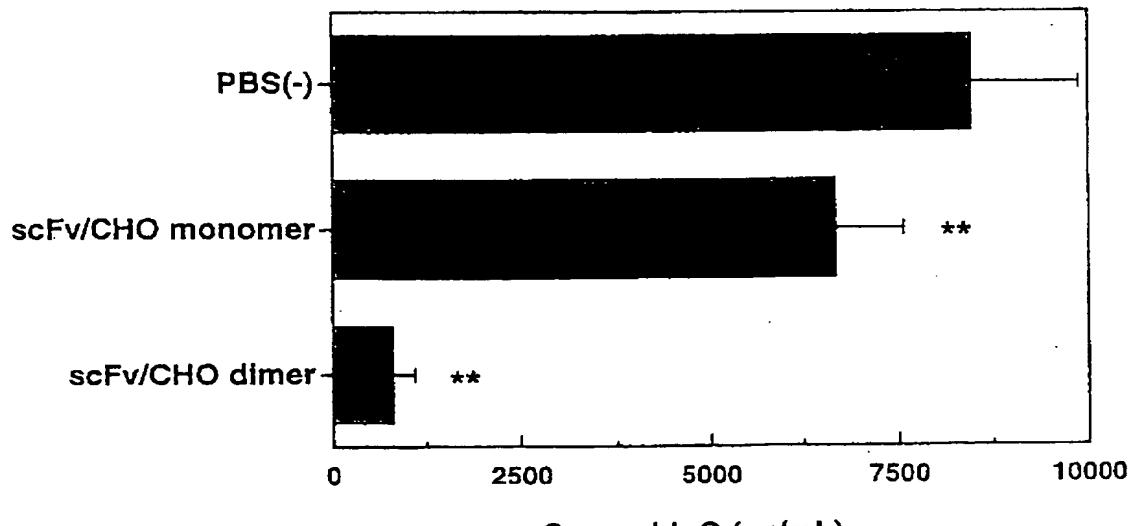


【図2-9】 Fig. 29



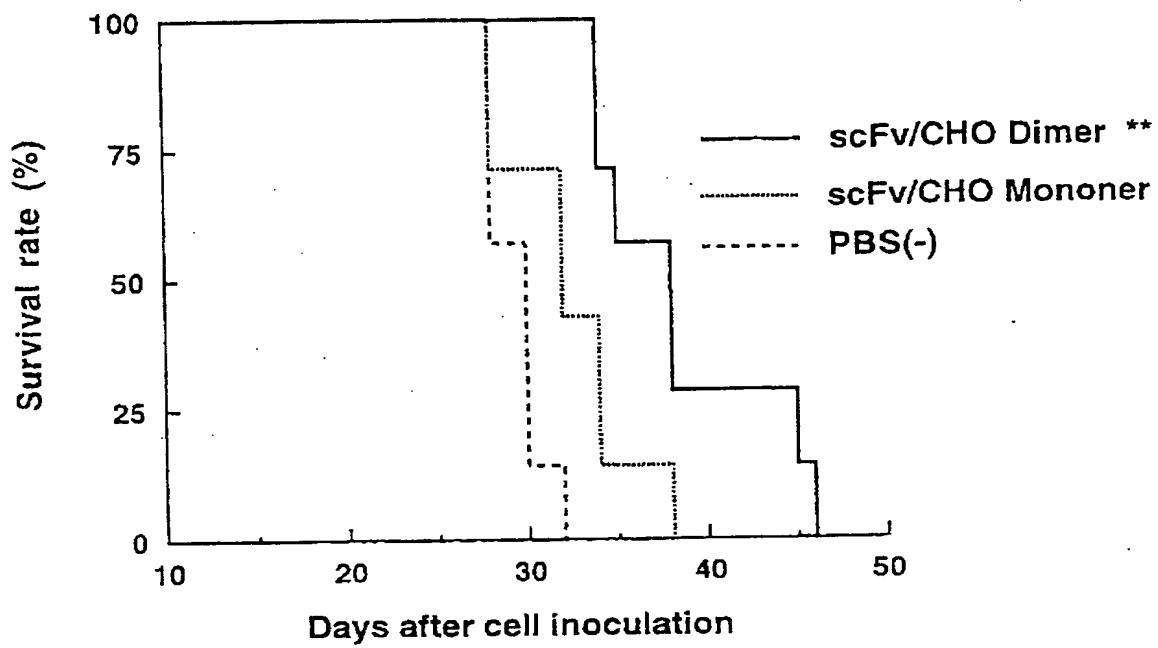
【図3-0】 Fig. 30

*Effect of MABL-2 (scFv) on serum hIgG in KPMM2 i.v. SCID mice*

\*\* :  $p < 0.01$

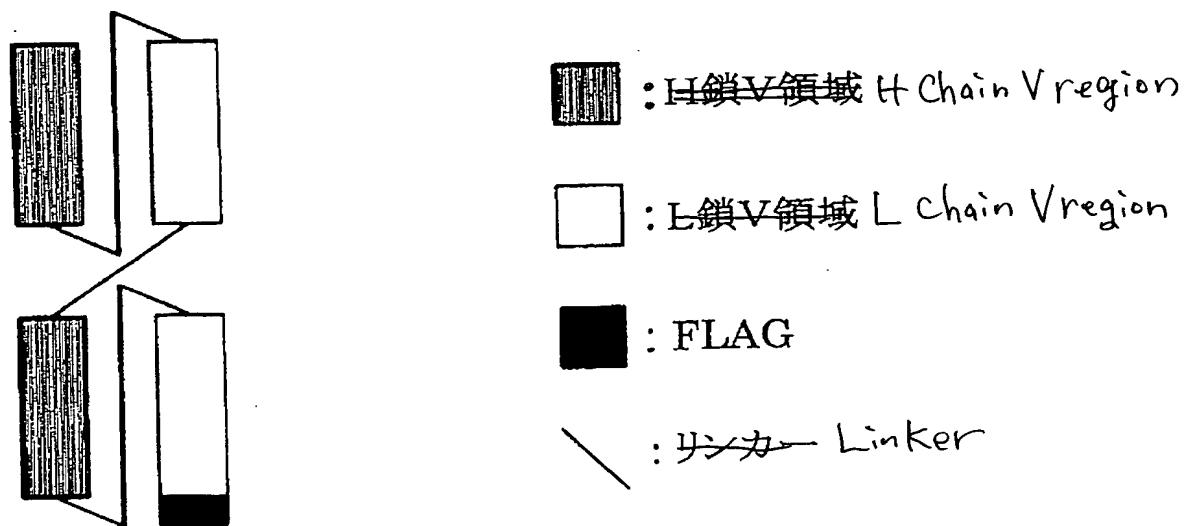
【図3-1】Fig.31

*Effect of MABL-2 (scFv) on survival  
of KPMM2 i.v. SCID mice*



\*\* ; P&lt;0.01 by t-test

【図3-2】Fig.32



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